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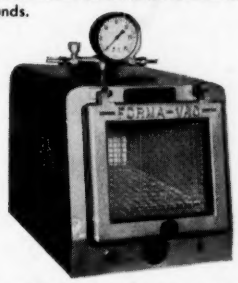

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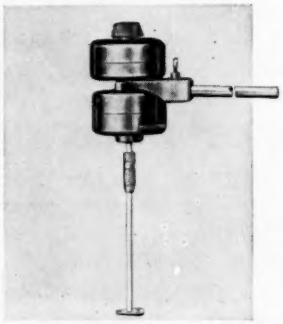

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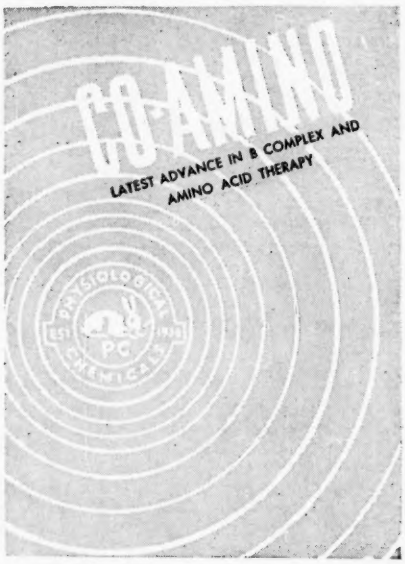

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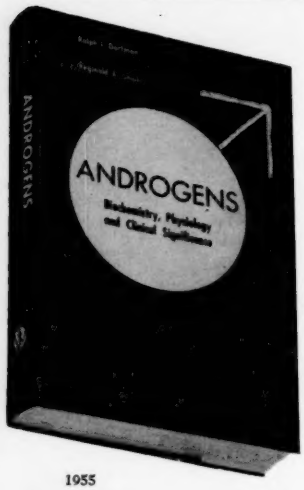

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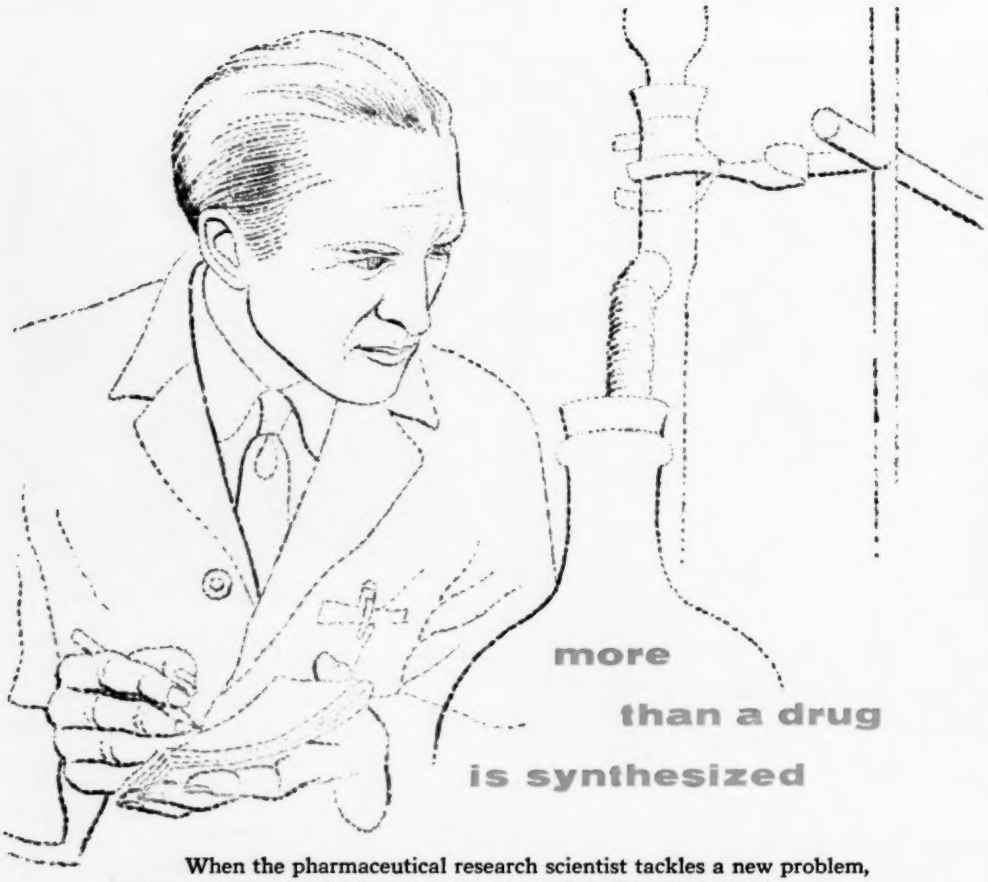

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LIPOPROTEINS IN PRIMARY BILIARY CIRRHOSIS[1]

By ELLA M. RUSS, JULIE RAYMUNT, AND DAVID P. BARR

(From the Department of Medicine, The New York Hospital-Cornell Medical Center, New York, N. Y.)

(Submitted for publication August 15, 1955; accepted September 7, 1955)

Evidence gradually accumulated by moving boundary electrophoresis (1–4), chemical fractionation, (5–7) using method of Cohn and his associates (8), zone electrophoresis in a starch-supporting medium (9), and the ultracentrifuge (10) has indicated that plasma lipids in the acute stage of infectious hepatitis, in biliary cirrhosis, and in some cases of biliary obstruction are atypically combined with peptides in the form of abnormal lipoproteins.

This is a report of an investigation to explore further by chemical, physical-chemical, and immunochemical means the composition and distribution of abnormal lipoproteins in a case of primary biliary cirrhosis and to compare their properties with lipoproteins from plasma of a normal woman of like age.

METHOD

Plasma was first fractionated according to method number 10 of Cohn and his associates (8) with minor modifications adapted by this laboratory (11). Three lipoprotein-bearing fractions, Fractions IV + V, VI, and I + III were obtained from biliary cirrhotic plasma. No lipoproteins are recovered in Fraction VI of normal plasma (8). Alpha and beta lipoproteins designated also respectively as high and low density lipoproteins were separated from all fractions and unfractionated plasma by ultracentrifugation in a Spinco Model L preparative ultracentrifuge after their solution densities had been adjusted to 1.063 − 1.068 with sodium chloride. The ultracentrifugally separated low density ($p < 1.063$) lipoproteins were further separated in a density gradient tube as recently described by Oncley and Mannick (12) and chemically characterized. Immunochemical examinations were made of Fraction IV + V, VI, and I + III, and ultracentrifugal analyses for S_f components were carried out on both unfractionated plasma and the fractions.

Preparation of unfractionated plasma. Five ml. of biliary cirrhotic ACD[2] plasma and 9 ml. of normal ACD[2] plasma were dialyzed against several changes of citrate-sodium chloride reagent[3] for 48 hours at 0° C. Densities of the dialyzed solutions were adjusted to approximately 1.065 with 4.0 M NaCl and distilled water until a final volume of 11.0 ml. was obtained for the biliary cirrhotic and 13.0 ml. for the normal plasma. Nine-ml. aliquots of each were placed in 13 × 85 mm. plastic centrifuge tubes and ultracentrifuged at 30,000 rpm (80,000 G) for 18 to 20 hours at 5° C. The top 3.0 cm. of solution were separated with a tube-slicing device. From the upper and lower cuts containing low and high density lipoproteins, respectively, 100 λ samples were removed for determination of solution density. The upper cut of the biliary cirrhotic plasma was diluted to 6.0 ml. with 2.3 M NaCl and from the normal plasma with 2.0 M NaCl. An aliquot of 4.5 ml. was removed from each into 13 × 85 mm. plastic tubes for density gradient studies (described presently); the remainder was dialyzed together with both lower cuts (high density lipoproteins) against 0.15 M NaCl for 24 hours at 0° C. The dialyzed solutions were then analyzed for lipids, nitrogen, and dry weight.

A linear density gradient as described by Linderstrøm-Lang and Lanz (13, 14) and Anfinsen (15) was made of the diluted upper cuts as follows: 4.5 ml. 0.1 M NaCl were layered over the 4.5-ml. aliquot of low density lipoproteins that had been previously placed in a 13 × 85 mm. plastic tube. A simply-constructed wire stirrer with a spiral button tip was gently inserted to the interface of the two solutions, from which point gentle vertical strokes of increasing 0.75 cm. distances were made, keeping the plane of the spiral tip as level as possible. The manipulation usually required four strokes each down and up from the interface. The tubes were then ultracentrifuged at 30,000 rpm for 18 to 20 hours at 5° C. Lipoproteins concentrate in such gradients at layers where the lipoprotein density is equal to the density of the sodium chloride solution. The resulting gradients were partitioned into 8 or 9 cuts, 0.75 cm. each, either by slicing with a tube-slicing device or by aspirating with a fine-tipped pipette. From the volume of each cut (approximately 1.0 ml.) a 100 λ sample was removed for determination of solution density. To the remainder of each cut was added an equal volume of 0.15 M NaCl; each

[1] These studies have been aided by Grant Number H-920 from the U. S. Public Health Service, by support from the Commonwealth Fund and the Albert and Mary Lasker Foundation, and by gifts from Mrs. Katherine Lilly Conroy.

[2] ACD (acid citrate-dextrose) is the special anticoagulant solution used when fractionating plasma proteins by the Cohn method (8).

[3] Citrate-sodium chloride reagent consisted of 0.02 M trisodium citrate and 0.07 M sodium chloride.

dilution was placed in Visking cellophane tubing (1 cm. in diameter) and dialyzed against 0.15 M NaCl for 24 hours at 5° C. The contents of each bag were removed as quantitatively as possible, the volumes measured, and the solutions analyzed for nitrogen, lipids, and dry weight.

Preparation of plasma fractions. In each of six tubes, 5-ml. samples of plasma (6 × 5 ml.) were fractionated simultaneously. Fractions from 1 × 5 ml. plasma were used solely for protein and lipid analyses as described in the quantitative method of Cohn and his associates (8).

Fraction IV + V obtained as a zinc proteinate paste from Fraction IV + V + VI of 3 × 5 ml. of biliary cirrhotic plasma or 5 × 5 ml. normal plasma were dissolved in 13.5 ml. of citrate-sodium chloride reagent and for complete removal of zinc ions dialyzed 24 hours at 0° C. against the same reagent (11). The density of the entire dialyzed solution was adjusted to 1.064 with 3 to 4 M NaCl yielding a final volume of 20.5 ml. From this mixture two 9-ml. portions were removed for ultracentrifugal separation of low and high density lipoproteins as described for unfractionated plasma. Absence of low density lipoproteins in normal plasma was ascertained by lipid analyses of the upper cut. The pooled upper cuts from biliary cirrhotic plasma were diluted to a volume of 12.0 ml. with 2.0 M NaCl, 4.5 ml. of which were removed for density gradient studies as already described; the remainder was aliquoted for (a) examination of S_f classes of lipoproteins (approximately 10 mg.), (b) immunochemical reactions, and (c) analytic purposes. The lower cut pools and aliquot (c) of the upper cuts were then dialyzed and analyzed for chemical components as described for unfractionated plasma.

Fraction VI, obtained as a supernatant of the Fraction IV + V paste just described for biliary cirrhosis, was concentrated by precipitation of the proteins with $NH_4Cl - NH_4OH$ buffer to a pH of 7.6 (16). The resulting paste was dissolved in 7.5-ml. acetate buffer $\Gamma/2 = 0.5$, pH 4.5 followed by dialysis against the same buffer for 24 hours at 0° C. to remove zinc ions. The density of the entire solution was adjusted to 1.066 by addition of 4.0 M NaCl and distilled water so as to obtain a final volume of 20.0 ml. From this solution two 9-ml. portions were removed for analysis as described in Fraction IV + V.

Fraction I + III, obtained as a paste from the Cohn fractionation of 3 × 5 ml. biliary cirrhotic plasma or 5 × 5 ml. normal plasma, was dissolved with 2.0 M NaCl and the density adjusted to 1.064 with distilled water and 2.0 M NaCl until a final volume of 20.0 ml. was obtained. From this solution two 9-ml. aliquots were removed for analyses as described for Fractions IV + V and VI.

ANALYTICAL PROCEDURES

Unfractionated plasma and all Cohn fractions were analyzed for concentrations of cholesterol, phospholipids, and protein and for distribution of electrophoretic components (moving boundary and filter paper methods) as previously described (8, 11, 17).

In addition, the following lipid procedures were employed. Micro or semi-micro adaptations were applied to the *free and esterified cholesterol* method of Sobel and Mayer (18) and *phospholipid* method of Stewart and Hendry (19). *Acetal phospholipids* by the Korey-Wittenberg procedure (20). *Total fatty acids* by variation of a standard gravimetric method as follows: 3:1 alcohol-ether extracts of plasma and fractions were evaporated to dryness in a 125-ml. soil digestion flask and saponified by refluxing gently with 10 per cent KOH in 95 per cent alcohol for one hour. The contents were cooled, diluted with water to bring the alcohol concentration to 40 per cent, transferred to a separatory funnel, and extracted three times with petroleum ether to remove the unsaponified material. The remaining soaps were acidified with concentrated HCl to liberate fatty acids which were then extracted three times with petroleum ether. The extracts were centrifuged to remove any traces of insoluble matter, quantitatively transferred to a tared flask, evaporated to dryness and desiccated to constant weight.

Fatty acids as neutral fat (triglycerides + free fatty acids) were estimated by subtracting the fatty acids of the predetermined ester cholesterol and phospholipids from the gravimetric value for total fatty acids according to the formula

$$\text{NFFA} = \text{TFA} - \{\text{ester cholesterol} \times 0.72 + \text{Lipid P} \; [0.8 \times 17.9 + 0.2 \times 8.95]\}$$

Neutral fat was calculated by multiplying the NFFA value by 1.05. In these calculations it is assumed that 80 per cent of the phospholipids exist as lecithin and cephalin and 20 per cent as sphingomyelin, that the average molecular weight of a fatty acid chain in ester cholesterol, phospholipid, and neutral fat is 277, and that the neutral fat contains three fatty acid chains.

Nitrogen analyses were made by a modification of the Conway (21) diffusion system following digestion with 0.5-ml. acid digestion mixture [4] in special micro Kjeldahl flasks.[5] The digest was diluted to 10 ml. with water and 0.5-ml. aliquots were removed for ammonia determination in 45 mm. Conway dishes. One-ml. saturated potassium metaborate was used to liberate the ammonia into 0.2 ml. 2 per cent boric acid which in turn was titrated with 0.005 N HCl. Concentrations of lipoprotein in solution were estimated from *dry weight measurements* according to Armstrong, Budka, Morrison, and Hasson (22) using the following variation. Protein solutions were dialyzed 48 hours at 0° C. against several changes of 0.15 M NaCl. Micro aliquots of the dialyzed material and final dialysate were dried to constant weight *in vacuo* at 38° C. for one week. The weight of protein was obtained from the difference in weight between the protein solution and that of the dialysate.

Densities were determined by weighing protein solu-

[4] Acid digestion mixture consisted of a 1:1 mixture of concentrated H_2SO_4 and saturated $KHSO_4$ containing 0.2 per cent $CuSO_4$.

[5] Micro Kjeldahl flasks, graduated to 5.0 and 10.0 ml.

TABLE I

Lipid analysis of plasma and lipoprotein fractions in biliary cirrhosis compared to normal

	Concentration as mg./100 ml. plasma in										
	Unfractionated plasma			Fractions							
				IV+V+VI			IV+V	VI	I+III		
	11/29 B.C.	1/5 B.C.	Norm.	11/29 B.C.	1/5 B.C.	Norm.	1/5 B.C.	1/5 B.C.	11/29 B.C.	1/5 B.C.	Norm.
Cholesterol, Total (Bloor)	1,620	1,565	196	1,223	1,300	53.4	443	780	237	264	134
Phospholipids	3,480	3,496	226	2,786	2,896	96.8	1,206	1,580	413	456	112
C/Pl RATIO	0.47	0.45	0.87	0.45		0.55	0.37	0.49	0.58		1.19
Cholesterol, Free (S-S)	1,595	1,750	44	1,232	1,440	10.0			165	192	34
Cholesterol Ester (S-S)	286	235	215	94	0	57.0			136	176	177
F/TC RATIO	0.90	0.93	0.26	0.96	1.0	0.23			0.67	0.68	0.24
Fatty Acids, Total		2,920	330		2,095	143.0				755	212
Neutral Fat (FFA + Trigl.)		626	120		253	63.0				427	71
Total Lipids		6,107	605		4,589	227.0				1,245	394
Acetal Phospholipids								0			

tions and water of same temperature in 100 λ pycnometers [6] on a micro analytic balance. For approximation of density as in adjustment of solution densities prior to ultracentrifugal separation of low and high density lipoproteins, a specific gravity spindle [7] was employed.

Analyses of S_f classes of lipoproteins [8] were made of the low density lipoproteins which were floated from plasma and concentrated fractions after dialysis against a solvent density of 1.063. All samples were then analyzed according to the method of Gofman and his associates (23, 24), using a Spinco Model E analytic ultracentrifuge.

Immunochemical analyses [8] of the lipoprotein fractions were carried out by the precipitin reaction described by Kabat and Mayer (25) using an antiserum which reacts with all low density lipoproteins from normal plasma as described by Levine, Kauffman, and Brown (26).

RESULTS

Lipid and protein concentrations in plasma and fractions

In Table I, concentrations of lipids in unfractionated plasma and Cohn fractions from primary biliary cirrhosis are compared with values of a normal woman. Immediately apparent is the unusual and characteristic distribution of the enormous quantities of the plasma lipids. Approximately 75 per cent of the total lipids and total fatty acids are recovered in Fraction IV + V + VI, where the cholesterol-phospholipid ratio is normal but the cholesterol is almost entirely unesterified. Inspection of the remaining plasma lipids recovered in Fraction I + III reveals a cholesterol-phospholipid ratio of 0.58 or about half of the ratio normally found for this fraction; the cholesterol is approximately 33 per cent esterified, and about two-thirds of the plasma neutral fat is recovered herein. Fraction VI contains approximately one-half of the plasma cholesterol and phospholipid, with only 91 mg. per cent protein as measured by the biuret method. Normally, only traces of lipids and approximately 50 mg. per cent protein are found in Fraction VI. No acetal phospholipid is detected in Fraction VI. The wash from Fraction I + III of any occluded Fraction IV + V + VI is normally devoid of lipids but in the biliary cirrhotic plasma 117 mg. per cent phospholipids and 145 mg. per cent total fatty acids were recovered.

Electrophoretic studies

Moving boundary electrophoretic patterns of unfractionated plasma and Cohn fractions in biliary cirrhosis are compared to a normal in Fig-

[6] 100 λ pycnometers obtained from Micro Specialties Co., Berkeley, California.

[7] Special micro spindles having a displacement of 5.0 ml. in a 10 ml. cylinder obtained from R. C. Ewald Co., Middle Village, L. I.

[8] We are indebted to Dr. Ray K. Brown, Division of Laboratories and Research, New York State Department of Health, Albany, N. Y., for these determinations.

ure 1. In biliary cirrhosis we find, as others have, a marked decrease in plasma albumin and an increase of beta-1, beta-2, and gamma globulin peaks. By fractionation we are able to demonstrate unusual appearance of a beta-2 peak in Fraction IV + V; and a moderate increase in beta-1 and beta-2 peaks in Fraction I + III and in gamma globulin in Fraction II.

Filter paper electrophoretic analyses of the plasma and Cohn fractions in biliary cirrhosis indicate that lipids of Fraction IV + V and I + III migrate with both beta-1 and beta-2 globulins, while in Fraction VI a general dragging of lipids is apparent from the origin through the beta-2 globulin area.

Density gradient analyses

In Figure 2, results obtained from analysis of each cut in the density gradient of low density ($p < 1.063$) lipoproteins floated from unfractionated plasma of a normal and a biliary cirrhotic are plotted against respective solution densities. In this and in Figures 3 and 4, weight ratios are given for cholesterol to phospholipids (C/PL)

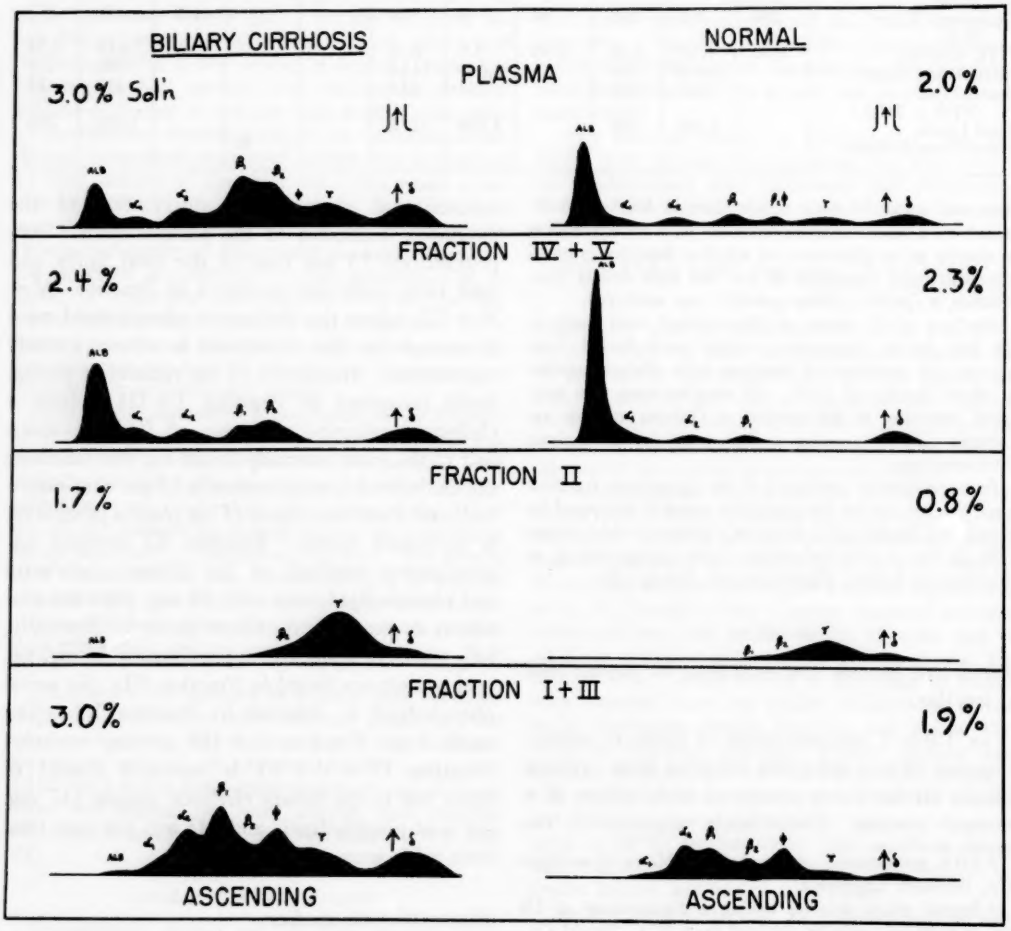

FIG. 1. ELECTROPHORETIC COMPONENTS IN UNFRACTIONATED PLASMA AND COHN FRACTIONS IN PRIMARY BILIARY CIRRHOSIS COMPARED TO NORMAL

Analyses were made of protein solutions differing in concentration as indicated to the sides of each pattern. Veronal-citrate buffer pH = 8.6, $\Gamma/2 = 0.1$, T = 2° C.

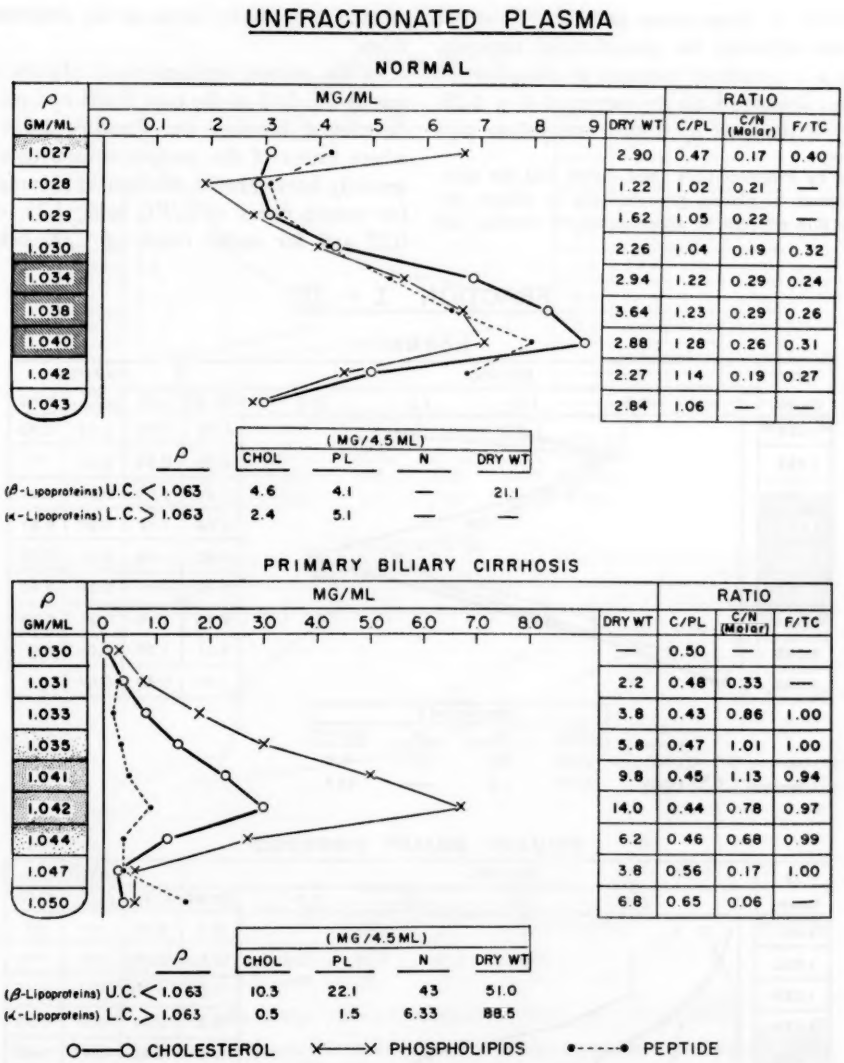

FIG. 2. ANALYSES AND PATTERNS OBTAINED FROM DENSITY GRADIENTS OF LOW DENSITY LIPOPROTEINS IN UNFRACTIONATED PLASMA OF A NORMAL AND A PRIMARY BILIARY CIRRHOTIC

ρ represents solution densities.

and free to total cholesterol (F/TC) and molar ratios for cholesterol to nitrogen (C/N). Also shown are analyses of (a) upper cuts designated as (U.C.) containing all low density ($p < 1.063$) or beta lipoproteins from which density gradients were made; and (b) lower cuts (L.C.) containing all high density ($p > 1.063$) or alpha lipoproteins if any were present.

It should be borne in mind that the first cut could contain unbound lipid such as triglycerides and that in the last cut there could exist lipid free proteins or possibly lipoproteins of which the fatty acids may have undergone oxidation, thus giving rise to heavier components. Such factors could influence observed densities and dry weight values presented as an estimation of the

concentration of lipoproteins per cut. Nitrogen figures are corrected for phospholipid nitrogen, assuming a 1 : 1 ratio of nitrogen to phosphorus [9] Peptide is calculated from the corrected N × 6.25. Turbidity is depicted by dotted and yellow pigmentation by shaded areas on the diagrams of the tubes.

In the normal unfractionated plasma approximately one-half of the total lipids and peptide are distributed between the densities 1.034 – 1.041 where ratios of the various constituents are essentially homogeneous throughout: average values for weight ratios of C/PL being 1.25, of F/TC 0.27 and for molar ratios of C/N being 0.26.

[9] Studies by Petersen (27) have shown that the enormous increases in plasma phospholipids in biliary cirrhosis are due entirely to accumulation of lecithin and cephalin.

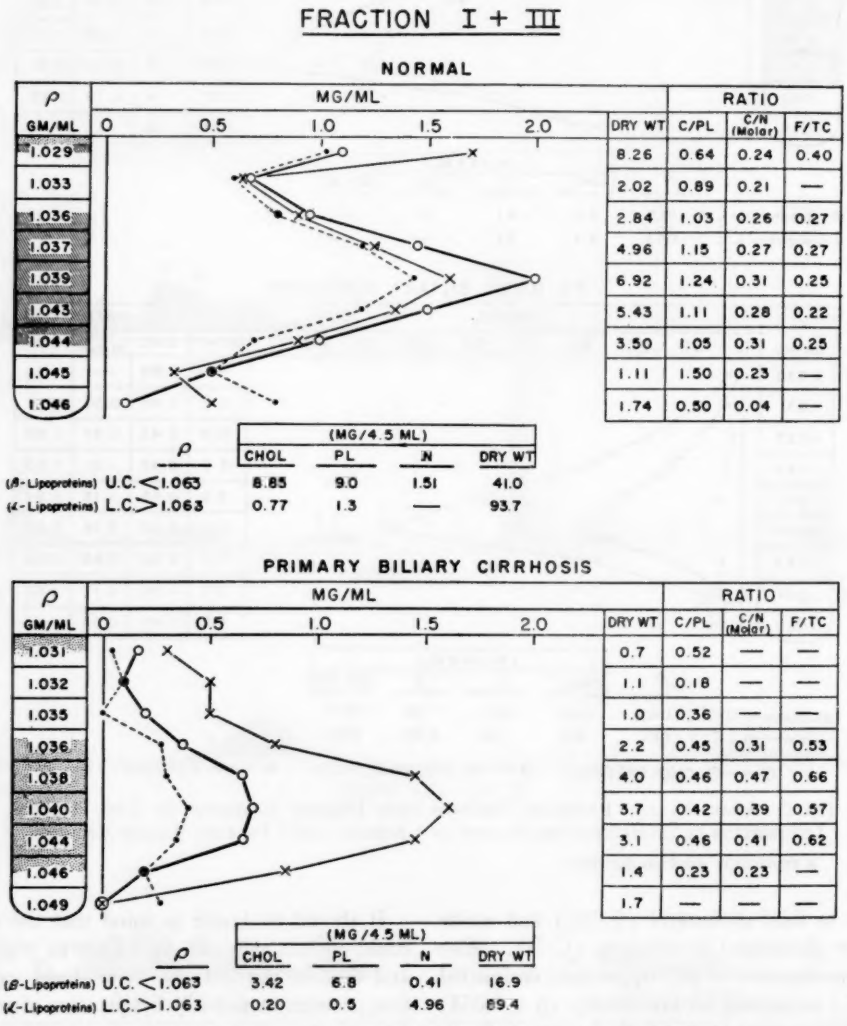

FIG. 3. ANALYSES AND PATTERNS OBTAINED FROM DENSITY GRADIENTS OF LOW DENSITY LIPOPROTEINS IN FRACTION I + III OF A NORMAL AND A PRIMARY BILIARY CIRRHOTIC

ρ represents solution densities.

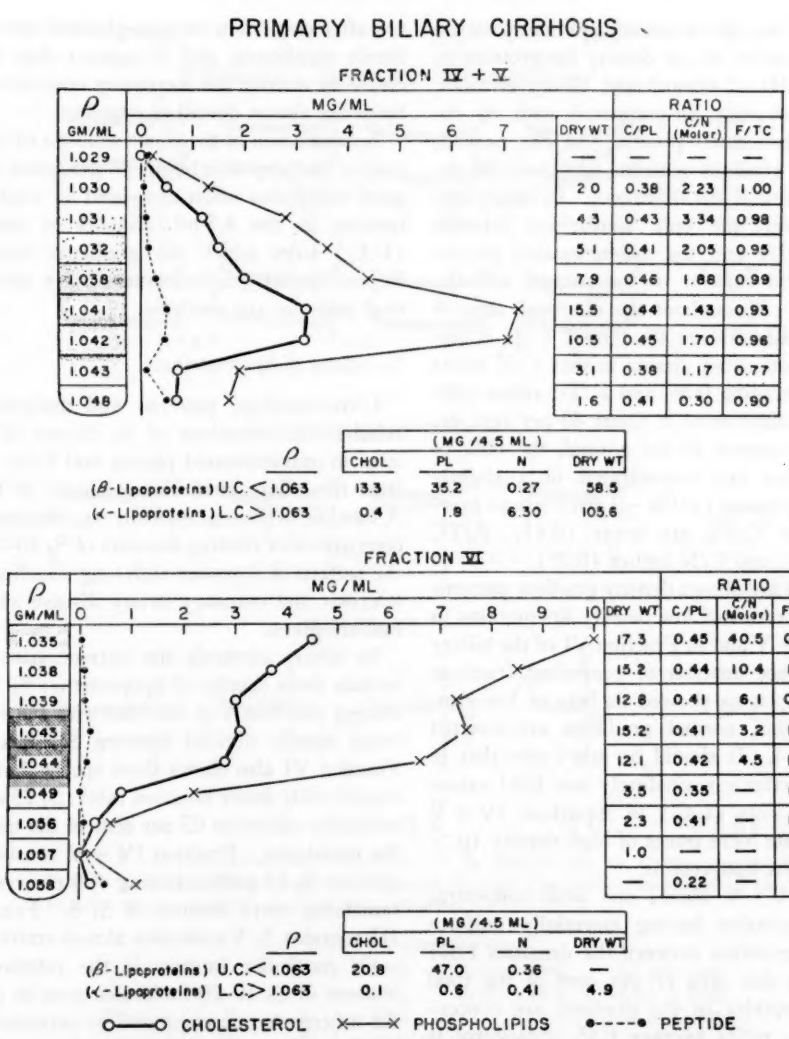

FIG. 4. ANALYSES AND PATTERNS OBTAINED FROM DENSITY GRADIENTS OF LOW DENSITY LIPOPROTEINS IN FRACTIONS IV + V AND VI IN A PRIMARY BILIARY CIRRHOTIC

ρ represents solution densities.

Studies from several other normals have shown that as much as three-quarters of the total lipoproteins in the gradient may be similarly distributed and that the contour of the patterns is the same. Outside the density range 1.034 – 1.041 the lipoproteins appear to have variable composition but appear to be similar in all the normals examined thus far.

In the unfractionated plasma of biliary cirrhosis as in the normal, about 70 per cent of the total concentration of lipids and peptide appear to pile up into one large peak between the densities 1.033 – 1.044, where the composition of lipoproteins is essentially uniform. In contrast to the normal in this range, however, the ratios for C/PL average only 0.45, for F/TC indicate little if any esterification of the cholesterol, and for C/N are much higher (.68 – 1.01).

In Figure 3 are shown density gradient patterns and chemical ratios of low density lipoproteins in Fraction I + III of normal and biliary cirrhotic plasmas. Each may be compared with its respective unfractionated plasma. In the normal, inspection of gradient patterns and analytic results reveals little if any difference. In biliary cirrhosis, however, the only similarities between Fraction I + III and the unfractionated plasma are the general contour of the pattern and the C/PL ratios. Throughout the one peak area of lipoprotein concentration Fraction I + III is unlike the unfractionated plasma in that C/N ratios are lower, averaging 0.36, and F/TC ratios indicate that the cholesterol is about 40 per cent esterified. In contrast to the normal, the bulk of the lipoproteins are concentrated in a slightly higher density range (1.036 – 1.046) where average ratios for C/PL are lower (0.45), F/TC higher (0.59), and C/N higher (0.39).

In Figure 4 are shown density gradient patterns and chemical ratios of low density lipoproteins in Fraction IV + V and in Fraction VI of the biliary cirrhotic. Since analysis of equivalent fractions from normal plasma showed no beta or low density lipoproteins, normal gradients are omitted from Figure 4. It should be noted also that in the biliary cirrhotic exceedingly low lipid values of the lower cuts (L.C.) of Fractions IV + V and VI indicate mere traces of high density ($p >$ 1.063) or alpha lipoproteins.

Fraction IV + V shows one peak concentration of lipoproteins having essentially uniform chemical composition between the densities 1.041 – 1.042. In this area 78 per cent of the total lipids and peptides in the gradient are concentrated, C/PL ratios average 0.45, cholesterol is almost entirely unesterified, and C/N ratios average 1.7.

In Fraction VI two peaks of lipid concentration are apparent; a peak in the density range 1.035 – 1.038 contains 46 per cent of the cholesterol and phospholipids of the entire gradient; the other in the range 1.039 – 1.049 contains 50 per cent of the total. In both, C/PL ratios are similar to those in Fractions IV + V and I + III. The cholesterol, however, is entirely unesterified. The C/N ratios in the density range $> 1.039 - <$ 1.049 are high (3.2 – 6.1). In the density range $<$ 1.035 – 1.038, however, the total nitrogen values after correction for phospholipid nitrogen are barely significant, and it appears that with decrease in density the increasing concentrations of lipids are almost devoid of peptides.

A summation of the concentrations of lipids, nitrogen, and dry weights in all gradients indicated good recoveries when compared to total concentrations in the 4.5-ml. aliquots of upper cuts (U.C.) from which the gradients were made. Repeat density gradient studies have shown identical patterns and analyses.

S_f classes of lipoproteins

Ultracentrifuge patterns and analysis of the relative concentrations of S_f classes of lipoproteins in unfractionated plasma and Cohn fractions from the biliary cirrhotic are shown in Figure 5. A baseline is photographically superimposed. For comparison of relative amounts of S_f 10–20 classes the pattern of a patient with long-standing atherosclerosis and coronary artery disease and a normal are given.

In biliary cirrhosis the unfractionated plasma reveals three species of lipoproteins: S_f 13 representing two-thirds of the total with the remaining being equally divided between S_f 10 and S_f 7. Fraction VI also shows three species but all have considerably faster flotation rates: S_f 25 and S_f 29 molecules comprise 65 per cent of the total, S_f 16 the remainder. Fraction IV + V consists of two species: S_f 13 predominating (90 per cent) and a remaining small amount of S_f 6. Fraction I + III contains S_f 5 molecules almost entirely.

Of particular interest is the relatively small amount of S_f 10–20 molecules seen in plasma of the atherosclerotic compared to exceedingly large concentrations of S_f 10 and S_f 13 molecules in the biliary cirrhotic.

Immunochemical analysis of fractions

Precipitin reactions were carried out on each of the fractions with antiserum reacting with all the low density lipoproteins of normal plasma. A positive reaction was obtained only for Fraction I + III.

DISCUSSION

The present study was carried out on plasma of a single case of xanthomatous biliary cirrhosis.

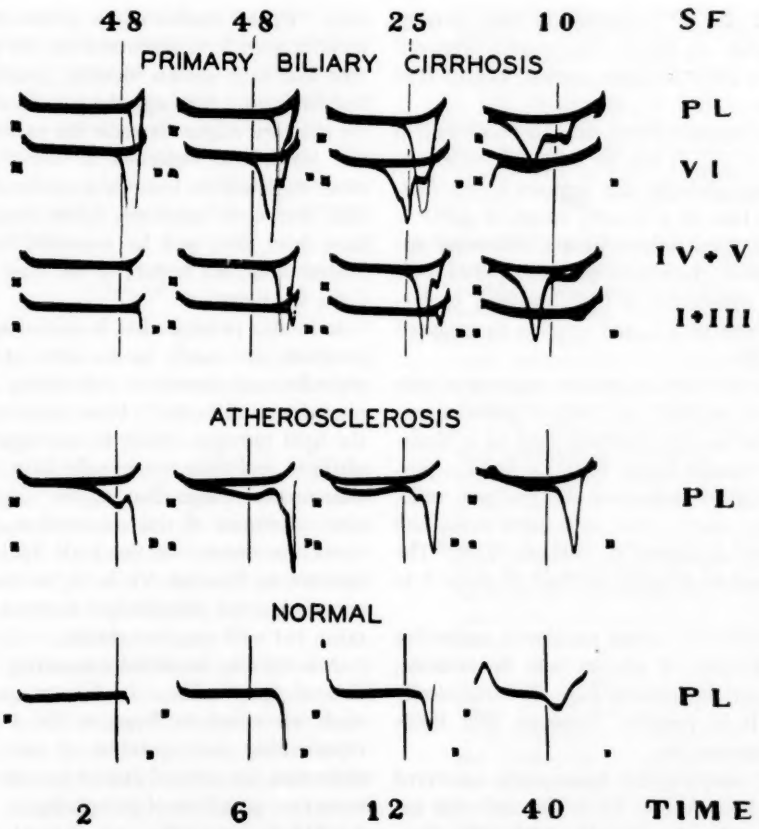

FIG. 5. ULTRACENTRIFUGAL ANALYSES OF S_f MOLECULES IN UNFRACTIONATED PLASMA AND COHN FRACTIONS OF A PRIMARY BILIARY CIRRHOTIC COMPARED TO THE PLASMA OF AN ATHEROSCLEROTIC AND A NORMAL

Primary Biliary Cirrhosis

Plasma	S_f 13 = 63%	S_f 10 = 19%	S_f 7 = 18%
Fraction VI	S_f 29 = 19%	S_f 25 = 46%	S_f 16 = 35%
Fraction IV+V	S_f 13 = 87%		S_f 6 = 13%
Fraction I+III	S_f 5 = approximately 100%		

Atherosclerosis

Plasma: S_f 20–74 = 31%; S_f 10–20 = 13%; S_f 2–10 = 56%

Similar observations on additional subjects might disclose minor variations. Analysis of plasma reveals only traces of alpha lipoprotein. Immunological tests indicate persistence of beta lipoproteins which are antigenically similar to normal beta lipoproteins but which account for only a small part of the enormous accumulation of plasma lipids. Most of the lipids are combined with peptides and with each other in atypical forms, the nature of which has been partially illuminated by the various physical chemical examinations of the plasma protein fractions.

It appears that there are in biliary cirrhosis at least three atypical lipoproteins or groups of lipoproteins. In each the lipoproteins migrate electrophoretically as beta globulins; the content of peptide in proportion to analyzed lipid is smaller than is found in normal beta lipoproteins; and mean density as observed in density gradient analyses is somewhat higher than Oncley and

Mannick (12, 28, 29) reported in their density gradient studies of beta-1 lipoprotein prepared from Fraction III − O from normal, pooled Red Cross plasma.

One of the atypical lipoproteins is recovered in Fraction IV + V. It has an electrophoretic mobility of a beta globulin and appears in the density gradient tube in a density range of 1.041 − 1.042. It is characterized by the following approximate ratios: cholesterol-phospholipid of 0.45, free to total cholesterol of 0.95, (molar) cholesterol to nitrogen of 1.5, and peptide to analyzed lipid of 1 to 50.

In Fraction VI there is another lipoprotein with electrophoretic mobility of a beta-2 globulin, appearing in the density gradient tube at a somewhat higher density range (1.043 − 1.044), possessing a similar cholesterol-phospholipid ratio, slightly higher free to total cholesterol ratio, and a much higher cholesterol to nitrogen ratio. The relation of peptide to analyzed lipid is about 1 to 80.

These lipoproteins do not react with antibodies to the protein part of normal beta lipoproteins, suggesting that the protein here is antigenically dissimilar. It is possible, however, that lipids block the antigenic site.

Neither of these atypical lipoproteins recovered in Fractions IV + V and VI is identical with another found in Fraction I + III. The major lipoprotein of Fraction I + III appears in the density gradient tube at a density of 1.040, where analyses in this range indicate a cholesterol to phospholipid ratio of 0.45, free to total cholesterol ratio of 0.63, molar cholesterol to nitrogen ratio of about 0.43, and a peptide to analyzed lipid ratio approximately 1 to 7. These values deviate widely from those of normal beta lipoproteins. Since normal beta lipoproteins occur in the same density range and since the immunological tests indicate the presence of beta lipoproteins antigenically similar to the normal, the possibility that the chemical analyses could represent mixtures must be considered. Thus the analytic data cannot be taken as definitive of the atypical lipoproteins in Fraction I + III.

In addition to these recognizable lipoproteins, there is in the density gradient analysis of Fraction VI a large accumulation of lipids at a density of 1.038 and less, which is almost devoid of peptides. Repeat studies of the plasma made several months apart have confirmed the low peptide content and have shown identical gradient patterns and analyses. Although the paucity of peptides in the mixture might discredit the existence of definite lipoprotein molecules in this density range, other explanations must be considered. It is possible that some extremely labile lipoproteins may have been destroyed by exposure to fairly concentrated alcohol buffers in the early steps of the Cohn fractionation.

It is also possible that in assuming that phospholipids are chiefly in the form of lecithin and cephalin and therefore calculating a nitrogen-phosphorus ratio of 1:1 we have overestimated the lipid nitrogen which is subtracted from total nitrogen and thus erroneously have derived peptide concentrations that appear scarcely significant. Because of this assumption and also because the density of the high lipid-low peptide mixture in Fraction VI is higher than might be expected, acetal phospholipid analyses were undertaken but with negative results.

A word may be added concerning the distribution of fatty acids. In biliary cirrhosis, fatty acids recovered in Fraction IV + V + VI and representing three-quarters of the plasma concentration are derived almost exclusively from the enormous quantities of phospholipids. In Fraction I + III, however, they are derived equally from the phospholipids and neutral fat (triglycerides + FFA). In normal plasma, the greatest concentration of total fatty acids is in Fraction I + III where they are derived equally from cholesterol esters, phospholipids, and neutral fats.

Difficulty is encountered in correlating peptide-lipid combinations detected by analysis of density gradients with the lipoproteins indicated by Gofman flotation rates. Density gradient separation of the lipoproteins floated from the unfractionated plasma in biliary cirrhosis fails to resolve the atypical lipoproteins, apparently because of general aggregation of lipoprotein molecules in the density range 1.035 − 1.044. Similar analyses made on the Cohn fractions afford closer inspection and better characterization. Study of flotation rates of lipoproteins in both unfractionated plasma and Cohn fractions provides additional properties. In biliary cirrhosis the major concentration of molecules is in the S_f 10–20 cate-

gory. Analytic data obtained from previous Cohn fractionation (5) and the present density gradient studies indicated, however, that the lipoproteins differ chemically from those in a disease such as atherosclerosis, which is also characterized by large concentrations of S_f 10–20 molecules. The discrepancy offers additional evidence that no one method of analysis suffices for characterization of lipoproteins.

SUMMARY

Lipoproteins in the plasma of a woman with biliary cirrhosis have been compared by chemical, physical-chemical, and immunochemical methods to those found in the plasma of a normal woman of like age.

Preliminary fractionation of plasma by Cohn method number 10 and subsequent examination of density gradients made of the lipoproteins floated from the fractions have permitted separation and analysis of several atypical protein-lipid combinations not distinguishable by examination of the unfractionated plasma.

In biliary cirrhosis only traces of high density or alpha lipoproteins are found. The presence of normal low density or beta lipoproteins can be inferred only from immunologic tests and if present account for only a relatively small part of the enormously augmented plasma lipids. Most of the lipids are combined atypically with beta globulins as low density lipoproteins.

Three separate abnormal beta lipoproteins have been partially characterized. In all of them the peptide in proportion to analyzed lipid is less than that found in normal beta lipoproteins. The cholesterol-phospholipid ratios are less than normal, and concentration of esterified cholesterol is low or even absent.

Gofman flotation analysis of plasma in biliary cirrhosis shows that molecules within the S_f 10–20 classes form the major portion of the spectrum and in far greater concentrations than seen in atherosclerosis. However, despite similar flotation rates, chemical analyses indicate the lipoproteins in biliary cirrhosis differ from those in atherosclerosis.

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AN ASOLECTIN ADSORBED SUBSTRATE FOR PROACCELERIN ASSAY

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In this report, evidence is presented that asolectin, a commercially available soybean lecithin, is capable under controlled conditions of specifically binding and removing proaccelerin from human plasma. To our knowledge no other selective binding agent or adsorbent of proaccelerin has been reported.

MATERIALS

Asolectin (commercially available from Associated Concentrates, Inc., 57–01 32nd Avenue, Woodside, New York) Lot Number 2085 was used in this investigation. Fifty mg. per ml. was suspended in distilled water with a Potter homogenizer in the amounts needed immediately prior to use.

Barium sulfate (Baker) analytical grade.

Adsorbed ox plasma. Ox blood freshly collected in one-tenth volume of 2.5 per cent potassium oxalate was centrifuged for 45 minutes at 2500 rpm (2° C.). The plasma was siphoned off and passed first through a 20 per cent asbestos, then through a 50 per cent asbestos filter by a technique previously reported (1).

Russell viper venom (Stypven, Burroughs Wellcome and Company, London, England) was diluted 1:40,000 in a 1:30 suspension of cephalin in veronal buffer as described by Hjort, Rapaport, and Owren (2), as was the technique for preparation of:

Prothrombin free ox plasma.

Normal human plasma was prepared from the blood of healthy persons. Following venipuncture with a 16 gauge needle, the first 5 ml. of blood was collected separately and discarded. The desired quantity of blood was then collected by gravity flow in a second glass bottle containing a quantity of 0.1 M sodium citrate solution equal to one-tenth the final volume, or when oxalated blood was necessarily used, into one-tenth volume of 2.5 per cent potassium oxalate solution. In either case, the freshly collected blood was centrifuged at 2500 rpm and 2°C. for 45 minutes. The plasma was transferred to another container with a Pasteur pipette and glass-activated by stirring for 5 minutes with one-eighth volume of fine quartz glass powder (particle size less than 0.07 mm.) by the technique of Rapaport, Aas, and Owren (3). Following centrifugation for 15 minutes at 2500 rpm and 2°C. to remove the glass powder, 20-ml. aliquots of the activated plasma were transferred to glass bottles and stored at minus 20°C. until they were thawed just prior to use.

Citrated, proconvertin deficient plasma was prepared from the blood of a patient with a congenital deficiency of this factor. Studies of the coagulation characteristics of this patient's plasma have been reported previously (4). Collection and processing were as described for citrated normal plasma. Aliquots kept at minus 20°C. were thawed just before use.

Citrated, proaccelerin deficient plasma was prepared in a similar manner from the blood of a patient with parahemophilia. The clotting characteristics of this patient's blood plasma have been reported in detail elsewhere (5).

Diluting fluid I was prepared as follows:

Sodium citrate 0.1 M solution	200 ml.
Sodium chloride 0.9 per cent solution q. s.	1200 ml.

Diluting fluid II was made by mixing:

Sodium citrate 25.66 mM solution	200 ml.
Veronal buffer	200 ml.
Sodium chloride 0.9 per cent solution q. s.	1000 ml.

Veronal buffer (pH 7.35, ionic strength 0.154) was prepared as previously described (2).

Buffer saline was prepared as follows:

Veronal buffer	100 ml.
Sodium chloride 0.9 per cent solution q. s.	1000 ml.

Calcium chloride aqueous solutions were prepared with the molarities indicated in each test system.

Saline extract human brain thromboplastin was prepared as described previously (6).

Thrombin: 1000 units of Hemoclaudan Adrenon Leo (Lövens Kemiske Fabrik, Köbenhavn) were dissolved in 10 ml. veronal buffer. Further dilutions were made with buffer saline to the extent noted in the text.

ASSAY SYSTEMS

All incubations and tests were performed at 37°C. in duplicate.

Fibrinogen reactivity:

Citrated control or test plasma, undiluted	0.2 ml.
Buffer saline	0.2 ml.
Incubate 3 minutes. Time from addition of:	
Thrombin (1:15 in buffer saline)	0.2 ml.

Thromboplastin time [Quick (7)]:

Citrated control or test plasma, undiluted	0.2 ml.
Thromboplastin	0.2 ml.
Incubate 3 minutes. Time from addition of:	
Calcium chloride 25 mM solution	0.2 ml.

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Proaccelerin assay:	
Test plasma (1:10 in diluting fluid II)	0.2 ml.
Parahemophilia plasma	0.2 ml.
Thromboplastin	0.2 ml.
Incubate 3 minutes. Time from addition of:	
Calcium chloride 30 mM solution	0.2 ml.
Proconvertin assay:	
Test plasma (1:10 in diluting fluid II)	0.2 ml.
Plasma congenitally deficient in proconvertin	0.2 ml.
Adsorbed ox plasma	0.2 ml.
Thromboplastin	0.2 ml.
Incubate 3 minutes. Time from addition of:	
Calcium chloride 35 mM solution	0.2 ml.
Prothrombin assay: [method of Hjort, Rapaport, and Owren (2)]	
Test plasma (diluted 1:5 in diluting fluid I; then 1:10 in diluting fluid II; final dilution, therefore, 1:50)	0.2 ml.
Russell viper venom (1:40,000 in suspension of cephalin 1:30 in veronal buffer)	0.2 ml.
Prothrombin free ox plasma	0.2 ml.
Incubate 3 minutes. Time from addition of:	
Calcium chloride 30 mM solution	0.2 ml.
Anti-thromboplastin detection:	
Test plasma, undiluted	0.2 ml.
Citrated, platelet poor, glass-activated normal plasma, undiluted	0.2 ml.
Adsorbed ox plasma	0.2 ml.
Thromboplastin	0.2 ml.
Incubate 3 minutes. Time from addition of:	
Calcium chloride 30 mM solution	0.2 ml.

PREPARATION OF PROACCELERIN DEFICIENT PLASMA FROM NORMAL PLASMA BY ASOLECTIN BINDING AND SEPARATION

To citrated, platelet poor, glass-activated, normal plasma, asolectin homogenized in distilled water was added in the amount of 25 mg. of asolectin for each ml. of citrated plasma. Sufficient additional distilled water was then added until the original volume of citrated plasma equalled one-sixth the final diluted volume. The glass container was swirled to disperse the asolectin suspension throughout the mixture, covered with Parafilm, and placed in a water bath at 37° C. for 2 hours. After this period, the dilute plasma-asolectin suspension was transferred to lusteroid tubes, capped, and spun in a Spinco preparatory ultracentrifuge at 40,000 rpm for 1 hour (2° C.). The tubes were removed with utmost care to avoid agitation of the contents. Caps were *not* removed since the cuff traps a small quantity of the original suspension. Instead, the central screw plug was removed (again without agitation of the tubes' contents) and a lumbar puncture needle fastened to a 20-ml. Luer-Lok syringe was carefully introduced to a point just above the bottom of the tube on the side opposite the sedimented asolectin. The clear dilute plasma was slowly aspirated until the thin floating surface layer of lipids approached the needle tip. Aspiration was then discontinued and the syringe contents transferred to a graduated cylinder where the clear dilute plasma from all tubes was pooled, measured, and transferred to a glass vacuum flask. The flask's contents were shell-frozen by spinning in a bath of methyl alcohol and carbon dioxide snow. Lyophilization was carried out. When dry, the plasma was reconstituted with distilled water equal to one-sixth the volume of the material transferred to the lyophilizing flask before drying. Correction of the pH to 7.3 was made by adding 0.5 N HCl dropwise with stirring. Multiple lyophilized samples were reconstituted simultaneously, pooled so that a single reference curve would serve for all, frozen and stored in 1-ml. aliquots at minus 20° C.

The assay results in Table I are representative for such reconstituted plasma:

It is seen that proaccelerin was almost completely removed, prothrombin concentration was slightly reduced, proconvertin activity less so. Fibrinogen reactivity was good. Anti-thromboplastic activity was not demonstrated.

The control plasma, without asolectin, was similarly diluted 1:6 with distilled water, alkalinized to pH 9.1 with 0.5 N NaOH, and incubated 150 minutes at 37° C. It was then centrifuged at 40,000 rpm for 60 minutes (2° C.), shell frozen, lyophilized, and reconstituted in distilled water equal to one-sixth the total dilute volume. The pH was corrected to 7.3 with 0.5 N HCl added dropwise. No comparable loss of proaccelerin in the reconstituted control plasma occurred as judged by its Quick thromboplastin time of 20.9 and 21.0 seconds in contrast to 75.0, 74.6 seconds for the asolectin adsorbed plasma similarly reconstituted.

It should also be noted that the reconstituted lyophilized asolectin adsorbed plasma was tested undiluted in the anti-thromboplastin detection test. This would actually represent a six-fold increase in concentration of any such substances in the clear supernate since reconstitution was effected with one-sixth the total dilute volume. Actually, no

TABLE I

The following assay results are representative for reconstituted asolectin adsorbed plasma

	Reconstituted asolectin adsorbed plasma *time in seconds*	Per cent	Unadsorbed plasma same source* *time in seconds*	Per cent
Thromboplastin time (Quick)	75.0, 74.6		11.9, 11.6*	
Prothrombin	39.8, 39.2	56	32.8, 32.3	72
Proaccelerin	81.6, 83.3	0	23.7, 23.2	100
Proconvertin	23.6, 23.2	70	22.6, 19.9	100
Fibrinogen reactivity	22.1, 22.9		20.7, 19.9	
Anti-thromboplastin detection	12.1, 11.7		11.9, 12.0	

* Same plasma diluted 1:6 with distilled water, pH adjusted to 9.1 with 0.5 N NaOH, incubated 150 minutes at 37° C., centrifuged 60 minutes at 40,000 rpm, shell frozen and lyophilized gave Quick thromboplastin times after reconstitution with distilled water equal to one-sixth of the total dilute volume and correction of pH to 7.3 with 0.5 N HCl of 20.9 seconds and 21.0 seconds on duplicate samples.

anti-thromboplastins were demonstrable even under these conditions.

Evidence that asolectin specifically binds proaccelerin rather than destroys it is seen in the following experiment: 25 mg. of asolectin per ml. of citrated normal plasma (platelet poor, glass-activated) was suspended directly in the plasma with a Potter homogenizer. This mixture was then transferred to a glass tube, covered with Parafilm and incubated in a water bath at 37° C. Periodically, samples were taken for testing in the following system:

Diluting fluid II	0.2 ml.
Thromboplastin	0.2 ml.
Homogenized asolectin in normal plasma (citrated, platelet poor, glass-activated) 25 mg. per ml.	0.2 ml.
Incubate 3 minutes. Time from addition of:	
Calcium chloride 30 mM solution	0.2 ml.

Incubation time in minutes	Time in seconds of duplicates
0	38.6, 39.2 (same plasma in same system without asolectin 14.2, 14.6 after 0 minutes incubation)
15	38.8, 39.4
30	40.4, 38.9
45	40.8, 39.2
60	40.6, 39.8
75	41.7, 39.1
90	41.2, 39.0
105	42.3, 39.5
120	41.6, 40.7

Plasma incubated with asolectin 25 mg. per ml. in fine suspension for 120 minutes, diluted 1:6 with distilled water, spun 40,000 rpm for 60 minutes to remove asolectin, lyophilized and reconstituted in original volume gave 88.0 and 87.6 seconds in the same system.

We interpret these results to mean that no considerable deterioration of proaccelerin occurs on incubation with 25 mg. per ml. asolectin for two hours at 37° C. We have previously demonstrated that dilution, alkalinization, incubation, centrifugation, lyophilization, neutralization, and reconstitution of normal plasma in the initial volume does not result in comparable loss of proaccelerin activity. The marked depletion of proaccelerin that occurs when asolectin is spun out reflects the specific binding and co-sedimentation of a proaccelerin-asolectin complex, in our opinion. Whether this binding is physical or chemical cannot be stated with certainty at this time. Further investigation along this line is planned as is further consideration of the inhibitory effect of asolectin on the thromboplastin time and whether this is related to the asolectin-proaccelerin complex (*i.e.*, an anti-proaccelerin effect). Reutilizing once used asolectin sediment in equal concentration to adsorb a second equal portion of dilute normal plasma by the identical technique results in marked reduction of its ability to remove proaccelerin. Reconstituted plasma adsorbed with once previously used asolectin gave proaccelerin reduction to only 60 per cent of original activity instead of 0 per cent as in the first adsorption.

The following curves (Figure 1) compare the proaccelerin assay of known dilutions of normal citrated plasma in diluting fluid I when parahemophilia plasma was the substrate (dotted line) and when reconstituted asolectin adsorbed plasma was the substrate (solid line).

Demonstration of the mutual inability of para-

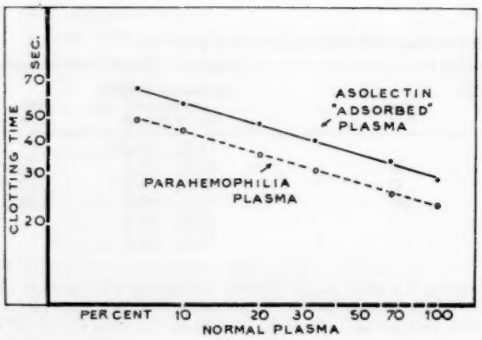

FIG. 1. COMPARISON OF PARAHEMOPHILIA PLASMA AND ASOLECTIN ADSORBED PLASMA AS SUBSTRATE FOR PROACCELERIN ASSAY

hemophilia plasma and reconstituted asolectin adsorbed plasma to correct one another is shown in the following results:

Parahemophilia plasma substrate	0.2 ml.
Reconstituted asolectin adsorbed plasma (1:10 in diluting fluid II)	0.2 ml.
Thromboplastin	0.2 ml.
Calcium chloride 30 mM solution	0.2 ml.
	81.6, 83.3 seconds (equals 0 per cent proaccelerin)

Reconstituted asolectin adsorbed plasma substrate	0.2 ml.
Parahemophilia plasma (1:10 in diluting fluid II)	0.2 ml.
Thromboplastin	0.2 ml.
Calcium chloride 30 mM solution	0.2 ml.
	87.6, 88.4 seconds (equals 0 per cent proaccelerin)

To determine if the results of the assay (Figure 1) were solely a reflection of the proaccelerin content and to exclude prothrombin and proconvertin activity as possible causes of the variations, the following experiment was designed:

Freshly prepared, oxalated, platelet poor, glass-activated, normal plasma was divided into two portions. One was mixed with 75 mg. of barium sulfate per ml. of oxalated plasma for 5 minutes at 20° C., then separated by centrifugation for 25 minutes at 2500 rpm (2° C.). Since some proaccelerin is non-specifically removed during barium sulfate adsorption, the unadsorbed oxalated plasma was diluted with buffer saline solution until its proaccelerin activity in correcting parahemophilia plasma substrate equalled that of the barium sulfate adsorbed plasma against the same substrate. The adjusted unadsorbed plasma mixture that accomplished this was 90 per cent oxalated unadsorbed plasma and 10 per cent buffer saline. Varying mixtures of barium sulfate adsorbed and unadsorbed plasma were tested in the proaccelerin assay system using asolectin adsorbed plasma as substrate. If this substrate is corrected by the proaccelerin content of the mixture and is insensitive to the varying prothrombin and proconvertin content, one would expect the same correction by all the mixtures. In Table II it can be seen that this was substantially the case. Test system is as described in section headed "ASSAY SYSTEMS" for proaccelerin with the difference that asolectin adsorbed reconstituted plasma is substituted as the substrate plasma for parahemophilia plasma.

COMMENT

Asolectin is not completely separable by centrifugation from undiluted plasma and, indeed, rises to the surface. Reduction of specific gravity and ionic strength by dilution with distilled water seems necessary for its complete separation by

TABLE II

Effect of varying prothrombin and proconvertin content on proaccelerin content in mixtures of $BaSO_4$ adsorbed plasma and unadsorbed plasma

Plasma mixtures						
Oxalated $BaSO_4$ adsorbed plasma (%)	100	80	60	40	20	0
Unadsorbed plasma (%)	0	20	40	60	80	100
Prothrombin (%)	0	20	40	60	80	100
Proconvertin (%)	0	20	40	60	80	100
Proaccelerin (%)	Constant in all mixtures					
Time in seconds	33.0	35.0	34.0	33.6	32.2	
Duplicates	32.6	34.8	33.6	33.0	33.6	

sedimentation on centrifugation. The adsorbed dilute plasma can then be lyophilized and reconstituted with an amount of distilled water equal to the original plasma volume.

Twenty-five mg. of asolectin per ml. of citrated plasma was determined to be the optimum amount and 120 minutes at 37° C. the optimum incubation period. Lesser amounts of asolectin or shorter periods of incubation resulted in less complete adsorption of proaccelerin. Greater concentrations of asolectin resulted in progressively larger adsorption of prothrombin in addition to proaccelerin and, with still greater concentrations, in considerable loss of fibrinogen reactivity.

One additional interesting thing becomes apparent from inspection of the systematic assay data (not presented here) when larger quantities of asolectin than recommended in this paper (for adsorption of proaccelerin) are used. Prothrombin adsorption becomes almost complete while proconvertin activity is only slightly diminished. Further examination of this phenomenon is discussed in another communication (8).

Paralleling their remarkable similarity of behavior in some other respects, we have found that cephalin derived from human brain by a modification of the technique of Milstone (9) also behaved as a proaccelerin adsorbent when substituted for asolectin in the technique outlined in this paper. Because asolectin is commercially available, whereas the preparation of cephalin from human brain is tedious, the technique using the former alone is reported in detail here.

It is interesting to speculate that possibly the similar lipid material of thrombocytes may be responsible for their demonstrated behavior as an adsorbent of proaccelerin (10).

SUMMARY

Evidence is presented that asolectin in an amount of 25 mg. per ml. of the original citrated plasma volume, suspended in normal plasma diluted 1:6 with distilled water, incubated 120 minutes at 37° C., and removed by centrifugation at 40,000 rpm for 1 hour, is capable of specifically binding and removing the proaccelerin of the normal plasma. The adsorbed dilute plasma was shell frozen, lyophilized, and reconstituted with one-sixth volume of distilled water. Control normal plasma similarly treated with the omission of asolectin exposure showed no comparable loss of proaccelerin.

Such asolectin adsorbed reconstituted plasma was comparable in its behavior as a substrate plasma for a proaccelerin activity assay system to the naturally occurring parahemophilia plasma used as a substrate plasma in the same system.

Evidence of the specificity of the asolectin adsorbed plasma substrate response for proaccelerin activity of the test samples is presented.

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A RELATION BETWEEN NON-ESTERIFIED FATTY ACIDS IN PLASMA AND THE METABOLISM OF GLUCOSE

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The concentration of non-esterified fatty acids (NEFA) in plasma increases after an injection of heparin (1). Apparently a lipase released into the blood (2, 3) hydrolyzes the neutral fat in chylomicrons (4) and promotes both the reduction of turbidity in the plasma and removal of fatty acids to tissues.

If the small amounts of NEFA normally present in blood arise from hydrolysis of circulating triglycerides, the concentration should remain constant or increase to some extent during spontaneous clearing of alimentary lipemia, depending on relative rates of liberation and removal. This expectation was not supported by the present work. In every case, after a mixed meal, NEFA fell sharply and remained below the fasting value for 2 or more hours; fat alone, when fed in sufficient quantity to produce gross lipemia, had no demonstrable effect on circulating NEFA. Feeding of glucose or injection of insulin caused a fall in concentration comparable to that occurring after the mixed meals, while the injection of epinephrine caused a marked rise.

METHOD

Inadequacies of existing techniques for measurement of NEFA made a new method desirable. The procedure described below is rapid and simple with about 3 per cent coefficient of variation on replicate analysis and a sensitivity sufficient for accurate titration of fatty acids in 1 cc. of plasma.

It appears to be quite specific for non-esterified fatty acids, at least with respect to other organic acids likely to enter plasma, but it cannot be regarded as absolutely reliable. For the present all that can be claimed is that NEFA are quantitatively extracted, that they represent by far the major part of the acidity and that the method can be used with the same confidence as has been given to the Davis method (5) or its various modifications (3, 6).

The extraction of lipides into a one-phase system and subsequent conversion of the system to two phases in the present method is similar in principle to the methanol-chloroform technique previously described by Folch, Lees, and Sloane-Stanley (7), and represents only a slight modification of the heptane-ethanol method developed in this laboratory by Blankenhorn and Ahrens (8). Heptane is a more convenient solvent than chloroform because it carries the lipides into an upper phase from which aliquot samples are easily taken; isopropyl alcohol, used in the present method instead of ethanol, is more efficient in bringing together heptane and water into a single phase. As a further simplification, titration of the heptane aliquot is conducted in a second two-phase system without preliminary evaporation of solvent.

Extraction mixture: isopropyl alcohol 40 parts; heptane 10 parts; 1 N H_2SO_4 1 part (all solvents redistilled). *Titration mixture:* 0.01 per cent thymol blue and 90 per cent ethanol in water, made by dilution of a stock 0.1 per cent thymol blue in water with 9 parts of redistilled ethanol. The titratable acidity of ethanol slowly increases over a period of several days, but is easily reduced to a minimum value by addition of alkali from the burette. A slight acidity, equivalent to about 2 λ of 0.018 N NaOH per cc. of titration mixture, is required to expel carbon dioxide from the system during titration. *Alkali:* approximately 0.018 N NaOH, made by 1/100 dilution of saturated NaOH with carbon dioxide-free distilled water. The alkali, protected with a soda lime column, is stored in a small reservoir mounted above a 0.100 cc. Rehberg burette. Fresh alkali, prepared each day of analysis, is calibrated by extraction and titration of standards of recrystallized palmitic acid in heptane.

As soon as possible after separation of plasma, 5 cc. of extraction mixture is added to 1 cc. of plasma in a glass-stoppered tube and shaken vigorously for a moment. After standing 10 minutes or longer the system is divided into two phases by mixing into it an additional 2 cc. of heptane and 3 cc. of water. The phases should separate rapidly without centrifugation, forming a sharp interface. A 3-cc. aliquot of upper phase is transferred to a 15-cc. conical centrifuge tube containing 1 cc. of titration mixture, and titrated with the alkali. Nitrogen, delivered to the bottom of the tube with a fine glass capillary, expels carbon dioxide from the sample and keeps the two phases mixed during titration. As the green-yellow endpoint is approached the gas stream is interrupted from time to time for examination of the indicator color in the alcoholic phase. Good lighting, such as that given by a fluorescent light placed just above and in front of the tube, has been found helpful in reading the endpoint.

In one test of reproducibility a series of standards, ranging from 0 to 713 μEq. per L., showed 2.6 per cent coefficient of variation between duplicate titrations of the same extract, and 3.6 per cent between titrations of separate extracts. The mean values fell on a straight line

TABLE I

Acidity of plasma fractions separated in the preparative ultracentrifuge at different densities *

Position	Density (D_4^{20})					
	1.01		1.07		1.20	
	C $\mu Eq./L.$	C/M	C $\mu Eq./L.$	C/M	C $\mu Eq./L.$	C/M
Top	104	0.07	178	0.20	180	0.42
Middle	1,215	0.86	760	0.86	406	0.95
Bottom					549	1.28
Pellet	5,090	3.58	lost		none	

* C = concentration of acidity determined with present method.

C/M = ratio of actual acidity to mean acidity of the whole tube. The first tube (1.01) contained untreated plasma. The second (1.07) and third (1.20) started with mixtures of plasma and 80 per cent sucrose in D_2O in the bottom and middle parts, and a protein-free layer of the same density (sucrose in D_2O; phosphate buffer, pH 7.4, μ 0.1) in the top. Centrifuged in a swinging bucket rotor 26 hrs. × 123,600 G at approximately 10° C.

passing through the origin, thus excluding bias in the method. The single extraction technique of the present method and the modified Davis extraction (5) described by Grossman, Stadler, Cushing, and Palm (6) gave nearly identical values in one experiment: single extraction 691 μEq. per L.; Grossman 702 μEq. per L. It will be noted that the comparison is limited to methods of extraction, since both extracts were titrated as described above; evaporation of solvent before titration removes most or all of the volatile short chain fatty acids, whereas they remain to be titrated in a two-phase system.

In further tests of the procedure the final concentration of mineral acid was varied from 0.01 to 0.10 N, the temperature was varied from 5° to 37° C. and the time was extended to 24 hours without significant changes in the titratable acidity of the heptane phase. Each of the following organic acids, extracted as described above, remained almost entirely in the aqueous phase; less than 2 per cent of the acidity added to any system could be recovered by titration of the heptane phase: α-ketoglutaric, β-hydroxybutyric, indole acetic, succinic, glycerophosphate, citric, malic, ascorbic, pyruvic. On the other hand, incubation of plasma at 37° C. before extraction did increase the extractable acidity at a rate of about 35 μEq. per L. per hour, presumably by enzymatic hydrolysis of esterified fatty acids; the increase was prevented by addition of 0.1 M $CuSO_4$ or ethanol. Heparinized plasma showed the same concentration of NEFA as aliquots of serum from the same bleeding; since the plasma could be separated more rapidly heparin was used as an anticoagulant in most experiments.

On ultrafiltration of plasma through a collodion membrane (Schleicher and Schuell) only 5 per cent of the titratable acidity passed into the filtrate.[1] Fractionation of plasma in a preparative ultracentrifuge (Table I) showed a sedimentation of acidity approximating the calculated behavior of albumin. At the lowest density the acidity became concentrated in the pellet, leaving very little to be extracted from the creamy lipide-rich upper layer. Increasing proportions of acid were found in the upper layer at higher densities, but even at 1.20 the major part had moved to the bottom of the tube. During the 2 hours required to set up the experiment and the 26 hours of centrifugation at 10° C. extractable acidity increased about 20 per cent; presumably the additional material became associated with protein in the same way as that originally present in the fresh plasma. The evidence from these experiments supports the belief that most of the acidity determined by the present method resides in albumin-bound fatty acids and that little or no hydrolysis of triglyceride occurs in the extraction or titration.

RESULTS

Non-esterified fatty acids of the plasma were determined in 82 subjects: 6 normal males, 57 male and female obese patients, 4 tuberculous women, 3 men with essential hyperlipemia, 3 children with active rheumatic fever, and 9 adult patients with various diseases (hyperthyroidism, mild diabetes, fever of unknown origin, sprue, bili-

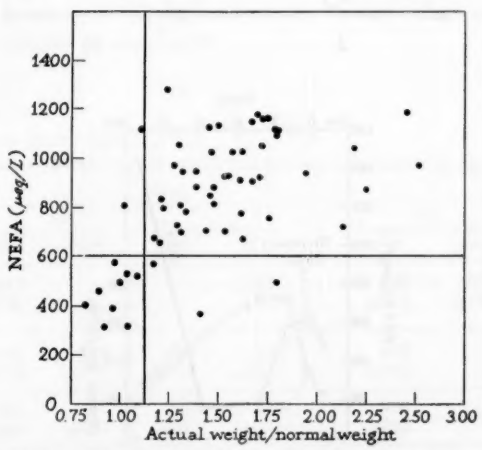


FIG. 1. FASTING LEVELS OF NON-ESTERIFIED FATTY ACIDS IN PLASMA RELATED TO THE DEGREE OF OBESITY, THE LATTER EXPRESSED AS A RATIO BETWEEN ACTUAL WEIGHT AND NORMAL WEIGHT FOR AGE AND HEIGHT (15)

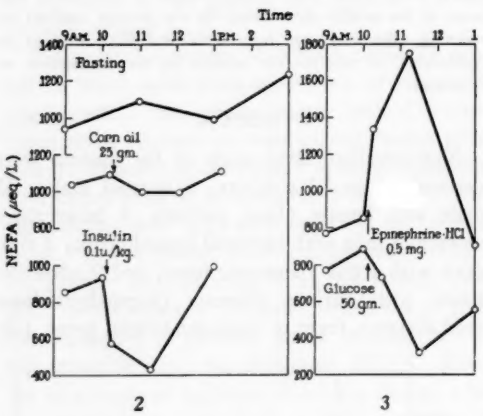
[1] Because almost all of the fatty acids in plasma are bound to protein it seems preferable to describe them as "non-esterified fatty acids," reserving the simpler term "free fatty acids" for the small but possibly important fraction actually free in solution.

TABLE II

Plasma lipides of three fasting hyperlipemic men

Patient	NEFA μEq./L.	Triglyceride fat		Cholesterol		Phospholipide	Total lipides
		mg. %	mEq./L.*	Free mg. %	Total mg. %	mg. %	mg. %
A	875	524	19.5	224	731	516	2,110
B	552	565	20.9	85	282	306	1,285
C	486	269	10.0	144	526	394	1,445
Normal range	500–900	300–400			150–250	150–250	600–1000

* Assuming the average equivalent weight of esterified fatty acid to be 270.



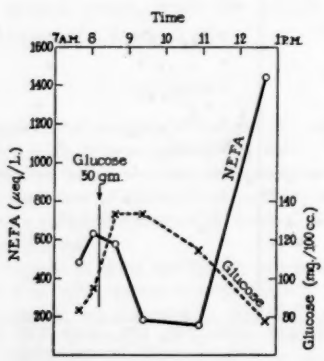


FIG. 2. EFFECTS OF FASTING (AVERAGE OF EIGHT EXPERIMENTS), CORN OIL (FOUR EXPERIMENTS) AND INSULIN (FOUR EXPERIMENTS) ON CONCENTRATION OF NON-ESTERIFIED FATTY ACIDS

FIG. 3. EFFECTS OF EPINEPHRINE (THREE EXPERIMENTS) AND GLUCOSE (14 EXPERIMENTS) ON CONCENTRATION OF NON-ESTERIFIED FATTY ACIDS IN PLASMA

FIG. 4. RECIPROCAL VARIATIONS OF GLUCOSE AND NON-ESTERIFIED FATTY ACIDS IN PLASMA AFTER INGESTION OF GLUCOSE

ary cirrhosis with marked reduction of liver function, Wilson's disease).

Fasting levels of NEFA, as determined in the morning after a 10 to 12 hour fast, ranged from 315 to 1210 μEq. per L. Obese subjects tended to have high concentrations at the beginning of the day; for example, the fasting level exceeded 600 μEq. per L. in 94 per cent of overweight people (ratio of actual weight to normal weight greater than 1.12), but reached this figure in only 18 per cent of those who were thinner (Figure 1). Excluded from this comparison are 2 patients with hyperthyroidism, 2 others with chronic low fever of unknown origin and 1 with sprue; these patients also showed high values, averaging 1100 μEq. per L. On the other hand, 3 hyperlipemic men had normal levels of NEFA despite high concentrations of other lipide fractions in their plasmas (Table II).

With continued fasting the concentration of NEFA rose slowly (Figure 2). By 3 PM a group of 8 obese women reached an average level of 1240 μEq. per L., while 4 thin women averaged 930 μEq. per L.—a concentration approximately equal to the morning average of the obese subjects.

A *mixed meal* eaten after a period of fasting invariably reduced the concentration of NEFA. One hour after a hospital breakfast, or lunch if breakfast had been omitted, the level had fallen from 850 ± 330 to 400 ± 160 μEq. per L. (12 patients, $p. < 0.01$). Following a single feeding of liquid formula (300 calories, of which 50 per cent from fat, 40 per cent carbohydrate, 10 per cent protein) (9) given to a group of 8 obese women, the NEFA fell from 1200 ± 240 to 790 ± 220 μEq. per L. ($p. < 0.01$).

Fat alone, when taken orally by fasting subjects, failed to increase the concentration of NEFA to any greater extent than fasting during the same period of time. Four obese subjects given 25 gm. of corn oil showed only a slight elevation or none at all, the maximum rise to 1245 μEq. per L. being less than that encountered in a number of fasting subjects. In two other experiments 50 gm. of corn oil, emulsified with 5 gm. of Ethofat 60/20 (Armour and Co.), caused marked lipemia with increase in the optical density of plasma at 600 mu from 0.06 to 0.37, but had no effect on the concentration of non-esterified fat (Table III).

Glucose, on the other hand, caused a marked decrease in level, 850 ± 220 to 290 ± 160 μEq. per L. ($p. < 0.001$), even greater than that seen after a mixed meal. A dose of 50 gm., given orally or intravenously, reduced NEFA to a minimum of approximately 200 μEq. per L. (Table III), reached about 1½ hours after ingestion and followed by a slow return to the initial value. Obese subjects, with higher fasting concentrations, showed the greatest changes in concentration, but in all cases the administration of glucose caused some reduction in level. Measurement of both glucose concentration and NEFA in three experiments showed a closely correlated reciprocal variation, illustrated by the curves in Figure 4. It will be noted that the amount of glucose administered in these experiments provided less than half the number of dietary calories that had produced no effect in the form of fat.

Insulin, like glucose, caused a sharp drop when injected subcutaneously in a dose of 0.1 μ per kg., whereas in three other experiments an injection of *epinephrine,* 0.5 mg., produced the highest levels encountered in the entire study (Table III, Figures 2 and 3).

DISCUSSION

In a recent abstract (10) Grossman, Moeller, Palm, and McDaniel reported that the concentration of non-esterified fatty acids in plasma rose from 500 to 700 μEq. per L. during periods of alimentary lipemia. The present results confirm the finding but cast some doubt on its relation to lipemia since comparable rises occurred when no fat was fed. In any case the changes in NEFA during fasting or after a fat meal were slight compared to the effects of glucose, insulin and epinephrine, and when both fat and glucose were given in a mixed meal the depressant effect of glucose predominated.

TABLE III

Non-esterified fatty acid in plasma (μEq./L.) *

Procedure	No. of expts.	Time							
		AM						PM	
		7:30	8:00	9:00	10:00	10:30	11:30	1:00	3:00
Fasting	8			943 ±240			1,085 ±231	996 ±242	1,236 ±196
Glucose 50 gm.	14			769 ±120	881 ±219	728 ±254	316 ±164	547 ±342	
Insulin 0.1 μ/kg.	4			854 ±161	933 ±272	567 ±400	438 ±135	988 ±268	
Epinephrine HCl 0.5 mg.	3			768 ±18	854 ±80	1,333 ±378	1,742 ±346	699 ±276	
Corn oil 25 gm.	4			1,037 ±108	1,089 ±168	999 ±115	998 ±162	1,112 ±134	
Corn oil 50 gm.	2	688 438	916 454	682 585	926 505		1,042 557	916 790	

* Test material was fed or injected immediately after the last bleeding to the left of the vertical line. Values in all rows but the last are the mean and standard deviation of experiments in the group; the last row shows the primary data of two experiments.

The responses of NEFA parallel the well-known effects of glucose (11), insulin (12), fasting (13) and epinephrine (14) on ketosis. Presumably both kinds of fatty acid—NEFA and ketone body—enter some metabolic process common to fat and carbohydrate.

SUMMARY

The concentration of non-esterified fatty acids in plasma increased with fasting, decreased after a mixed meal, oral glucose (50 gm.) or injected insulin (0.1 μ per kg.), and rose markedly after injection of epinephrine (0.5 mg.). Corn oil, on the other hand, failed to cause any change even when enough was taken to produce gross lipemia. The fasting levels of NEFA were normal in 3 men with chronic elevation of total plasma lipides, neutral fat, cholesterol and phospholipides.

A new method for determination of NEFA is described. It appears to be more rapid and sensitive than previous techniques.

ACKNOWLEDGMENT

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RESPONSE TO MERCURIAL DIURETICS DURING ALKALOSIS: A COMPARISON OF ACUTE METABOLIC AND CHRONIC HYPOKALEMIC ALKALOSIS IN THE DOG[1]

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A relationship between the action of mercurial diuretics and acid-base metabolism has been established for many years. Diuresis may be augmented by the administration of acidifying salts such as ammonium chloride (1), and may be decreased or even totally inhibited by alkalinizing agents (2, 3). Although a variety of clinical and experimental studies suggest that the mercurials act by decreasing the tubular reabsorption of the chloride ion (3–7), it is not clear exactly why this action of the drug is so susceptible to changes in acid-base balance.

Recent reviews have emphasized the role of glomerular factors in determining the response to mercurials. Potentiation by ammonium chloride has been attributed to an increase in the filtered chloride load, rather than to changes in the pH of the serum or of the urine (3). Conversely, it has been proposed that the refractoriness seen in metabolic alkalosis is due to a decrease in the amount of chloride filtered at the glomerulus (7). In addition, when the level of serum chloride is kept constant but the filtration rate is changed, the diuretic effect of the mercurials varies directly with the filtered chloride load (8).

It had previously been postulated that the pH of the tubular urine, or of the surrounding cells, might directly modify the dissociation of the mercurial and hence determine the extent to which mercury became affixed to the tubule (9). Although changes in the reactivity of the tubule to mercury have not been directly demonstrated, this possibility continues to be suggested by several observations which can not be explained solely by glomerular factors (*i.e.*, the filtered chloride load). Hilton (10) showed that the potentiation by ammonium chloride persisted in long term experiments after the elevated level of chloride in the serum had returned to normal. Since filtration rates were not measured, an increase in the amount of chloride filtered could not be excluded. However, it is clear that potentiation was not due to hyperchloremia alone. More striking discrepancies between the chloride load and the effectiveness of mercurials have been observed during acute hypochloremia. The chloruretic action of mercury may be completely abolished by infusions of sodium bicarbonate (3, 11) but not by infusions of sodium nitrate (6) which produce a comparable depression of the serum chloride level.

The acute administration of either acidifying or alkalinizing salts changes the pH of the calculated intracellular space in the same direction as it does that of the plasma (12). Presumably, the cells of the renal tubule respond in a similar manner. Thus, when large volumes of bicarbonate are infused, the resultant hypochloremia is accompanied by an increase in the pH of the extracellular fluid, of the tubular urine, and of the cells of the renal tubule. If the overall effect of the mercurials were dependent not only on the amount of chloride filtered but also on the pH of the tubules, these separate factors might not readily be dissociated by acute experiments with infusions of this type.

A possible method for elucidating the role of tubular pH was suggested by recent studies on the acid-base disturbance associated with potassium depletion. This is characterized by an extracellular alkalosis with hypochloremia, but the intracellular fluid becomes more acid. These changes have been demonstrated for skeletal muscle by several different techniques (13, 14) and for the renal epithelium both by indirect methods

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in the intact animal (15, 16) and by direct tissue analysis under *in vitro* conditions (17).

The present experiments were therefore undertaken to compare the effect of organic mercurial diuretics during acute metabolic alkalosis (extracellular and intracellular alkalosis) with their effect during potassium depletion (extracellular alkalosis and intracellular acidosis). The results clearly indicate that the diuretic response is not solely determined by the filtered chloride load, and, therefore, that tubular factors must play an important role.

METHODS

The effect of meralluride on electrolyte excretion was determined by clearance techniques in two trained female dogs which were depleted of potassium by the combination of a synthetic low potassium diet and the daily subcutaneous injection of 10 mg. of desoxycorticosterone acetate (DCA). Control studies in the pre-depletion period were made in dog B while on a stock diet of Kasco Dog Ration and in dog W while on the synthetic diet supplemented by 50 mEq. KCl per day. Venous blood was taken 2 to 3 times per week for estimation of plasma electrolytes. When the syndrome of alkalosis due to potassium depletion was fully developed, the effect of meralluride was again determined. The animals were then placed on a diet of raw hamburger and were given 20 mEq. per L. of KCl as drinking water; the administration of DCA was continued. The effects of meralluride were again determined after potassium repletion.

After the first period of potassium depletion dog B was placed on a stock diet for two months, and was then depleted again. In the second experiment, meralluride was given every two to three days while 1) on the synthetic diet with supplementary potassium, 2) on the same diet without potassium and 3) on the low potassium diet plus 20 mg. DCA per day. Electrolyte excretion was determined in daily specimens rather than for shorter collection periods.

The animals were kept in metabolism cages and the voided urine was collected daily. The volume, pH, specific gravity, and the concentrations of sodium, potassium, and chloride in the urine were determined each day. The feces were not measured. The daily balance was estimated as the difference between dietary intake (corrected for uneaten residues) and urinary excretion. Appropriate corrections were also made for the amount of electrolyte infused and excreted during the clearance studies. Water intake was not measured except during the period of potassium repletion when the KCl in the water was added to the calculated intake. No correction was made for the electrolytes removed in the blood samples. In calculating the cumulative balance, zero balance was assumed for the day on which the low potassium diet was started.

The electrolyte content of each diet was determined by analysis of nitric acid digests. The synthetic diet was (by weight): acid-washed casein 20 per cent, sucrose 44 per cent, hydrogenated vegetable fat (Crisco) 35 per cent, and Ruffex, 0.5 to 1.0 per cent. To 100 gm. of this diet were added 3.5 gm. of a supplementary salt mixture consisting of calcium, phosphate, magnesium, iron and trace elements (*cf.* 13). Vitamins were also added in adequate amounts. Dog B received 200 gm. and dog W 250 gm. of the diet per day. By analysis of different lots, the daily amount of electrolyte in the diet was less than 0.2 mEq. of potassium, less than 0.6 mEq. of sodium, and from 6 to 9 mEq. of chloride. To the first diet of dog B were added weighed quantities of $NaHCO_3$ and NaCl. Minor alterations were made during the course of the experiment, but the average intake was 100 mEq. of sodium and 50 mEq. each of chloride and bicarbonate per day. Dog W received 50 mEq. of KCl and 50 mEq. mono-sodium glutamate during the control period, and an equivalent amount of NaCl was substituted for the KCl during the depletion period. The glutamate was added in an attempt to make the diet more appetizing, but the results were equivocal. The second experiment on dog B was with a similar diet, *i.e.*, with glutamate added. In the first experiment, dog B had received an equivalent amount of sodium bicarbonate. The changes in electrolyte metabolism and the response to meralluride were the same in both instances.

Renal clearances were performed with standard techniques using the exogenous creatinine clearance as a measure of the glomerular filtration rate. The animals were studied in the fasting state but had been allowed free access to water. After the priming infusion, one-half hour was allowed for equilibration and then two control periods were taken. One ml. of meralluride (Mercuhydrin®, Lakeside Labs., Milwaukee; 39 mg. mercury per ml.) was injected intravenously during the course of five minutes. Observations were continued for five to six more clearance periods.

Blood was taken from the jugular vein in an heparinized oiled syringe. An aliquot was kept anaerobically under mineral oil for carbon dioxide determination; the blood was centrifuged promptly and the plasma separated. In experiments 5 and 6, urine was collected under oil without bladder irrigation, but in all other experiments the bladder was washed with 15 ml. distilled water at the end of each collection period. The failure to employ an anaerobic technique for the collection of urine introduced minor errors into the determination of carbon dioxide in the urine, but these were so small that this procedure was adopted since it assured more complete emptying of the bladder.

Plasma creatinine was determined on trichloracetic acid filtrates by the alkaline-picrate method; sodium and potassium by internal standard flame photometry; plasma carbon dioxide by the manometric method of Van Slyke; [8]

[8] The plasma bicarbonate concentration was calculated from the total CO_2 content assuming a constant pCO_2, except in the experiment in which plasma pH was directly determined.

urine carbon dioxide by either the volumetric or manometric method of Van Slyke; and chloride (first experiment dog B) by the method of Schales and Schales (18), and in the other experiments by a modified Volhard procedure.

RESULTS

The experimental plan and a summary of the results are shown in Table I. The effect of meralluride on electrolyte excretion was measured in a total of twelve clearance experiments while on the control diet, during potassium depletion, and after the potassium deficit had been corrected.

General course of potassium depletion

Each dog ate all of the synthetic diet and maintained body weight for 3 to 4 weeks, but thereafter food was gradually refused. The development of potassium depletion was associated with episodes of muscular weakness and paralysis which became progressively more severe. Both the anorexia and the asthenia promptly disappeared when the animals were potassium repleted. During the period of the low potassium diet, there was marked polydipsia and polyuria and the urine was

TABLE I

Summary of balance data and clearances showing chloruretic effect of meralluride during different types of alkalosis

Day	Wt.	Exp.	Remarks	Cumulative balance Na	Cumulative balance K	Cumulative balance Cl	Infusion*	Plasma† Na	Plasma† K	Plasma† HCO_3	Plasma† Cl	GFR‡	Change in chloride excretion§
	Kg.			*mEq.*			*mEq./L.*	*mEq./L.*				*ml./min.*	*mEq.*
						DOG B							
−6	14.6	1	Normal diet	—	—	—	None	145	4.4	23.5	115	47	+51
−3	14.6	2	Normal diet	—	—	—	$NaHCO_3$, 150	—	—	24.5	117		
								141	3.9	37.2	106	51	− 7
0	14.4		Start K free diet, DCA 10 mg./day.										
7	14.6	3		+ 10	− 36	+ 30	None	148	3.4	26.0	114	44	+57
15	15.0	4		+177	− 97	+ 27	None	144	2.7	25.6	112	45	+37
25	14.4	5		+198	−188	−135	None	148	2.3	33.1	105	45	+47
26			Start high K intake, maintain DCA 10 mg./day.										
35	13.6	6		+ 47	− 31	−103	$NaHCO_3$, 150	144	4.3	23.6	112		
								149	2.8	33.5	105	49	− 3
						DOG W							
−7	17.7	7	Normal diet	—	—	—	$NaHCO_3$, 150	149	4.3	23.2	110		
								140	3.0	33.0	100	53	+13
−3	17.3	8	Normal diet	—	—	—	NaCl, 120	149	4.3	23.2	111		
							$NaHCO_3$, 25	148	3.4	23.2	109	63	+41
0			Start K free diet, DCA 10 mg./day.										
23	16.8	9		+299	−220	+107	NaCl, 45	148	3.3	30.6	100		
							$NaHCO_3$, 100	154	2.5	38.1	101	31	− 3
30	15.0	10		+457	−271	+171	NaCl, 90	137	2.1	29.5	93		
							$NaHCO_3$, 31	139	1.9	31.9	95	57	+80
							KCL, 10‖						
31			Start high K intake, maintain DCA 10 mg./day.										
39	15.5	11		+484	− 69	+328	$NaHCO_3$, 150	151	3.2	24.9	110		
								152	2.5	35.9	100	60	+ 5
49	15.9	12		+557	− 11	+403	NaCl, 120	150	3.0	23.3	111		
							$NaHCO_3$, 25	150	2.5	24.1	112	73	+49

* Infusions contained creatinine in addition to the components indicated and consisted of 600 and 425 ml. priming for experiments 2 and 6, and between 500 and 550 ml. priming for experiments 7–12; sustaining infusions were about 4 ml. per min., except experiment 6 (2 ml. per min.). When no infusion is indicated, dog received only 3.5 per cent creatinine at less than 1 ml. per min.

† Values for plasma electrolytes (when given on single line) are averages of two periods before and two periods after administration of meralluride. When plasma values are given on two lines, the upper refers to the plasma level before the indicated infusion, and the lower refers to the average during the experiment, as calculated above.

‡ Glomerular filtration rate (GFR) is average of two periods before and three periods after administration of meralluride.

§ Change in urinary excretion of chloride is calculated as the amount of chloride excreted during 100 mins. after injection of meralluride corrected for the rate of chloride excretion during the two control periods before meralluride.

‖ KCl added to infusion of Exp. 10 in effort to prevent further depression of plasma K by dilution.

consistently acid (about pH 6). When the potassium deficit was corrected, even though the administration of DCA was continued, there was a significant reduction in daily urine volume with a rise in the specific gravity and pH of the urine. Although the findings suggest a direct effect of potassium on the capacity of the kidney to excrete a concentrated urine, further studies seem warranted to define more accurately the relative roles of electrolyte imbalance and steroid administration in the pathogenesis of this "diabetes-insipidus" syndrome (*cf.* 19, 20).

The concentrations of electrolytes in the plasma changed in accordance with the pattern that has been previously described for chronic potassium depletion (21). The plasma bicarbonate rose about 10 mEq. per L. and the chloride fell an equivalent amount. No consistent changes in the concentration of sodium were noted. The plasma potassium fell and remained in the region of 2 to 2.5 mEq. per L. In dog B at the height of potassium depletion (Exp. No. 5), the venous blood pH was 7.47 and the pCO_2 was within normal limits. This is in agreement with the extensive studies of Roberts, Randall, Sanders, and Hood (16) who found no change in pCO_2 during hypokalemic alkalosis. When the potassium intake was increased, the serum concentrations of bicarbonate, chloride and potassium were quickly restored towards normal.

Although the cumulative balance data (Table I) clearly demonstrate the development of negative potassium and positive sodium balances, it is probable that the values are subject to systematic errors. The careful studies of Howell and Davis (22) showed that large amounts of potassium were excreted in the feces of the dog given DCA; there was a significant fecal loss of sodium but this was less influenced by the steroid. In the present studies, fecal losses were not measured, nor was the nitrogen balance determined. These factors, combined with the inevitable losses in the metabolism cage, would indicate that the cumulative balances reported here are falsely high in a positive direction. However, it is probably of significance that the positive sodium balance was consistently much greater than the chloride balance. This suggests the migration of sodium to an intracellular site in exchange for potassium but further documentation by detailed calculations is not warranted by the present data. However, such intracellular changes have been demonstrated by tissue analyses in the dog after the production of an extracellular alkalosis by an experimental regimen similar to that employed here (23).

Effect of meralluride

During each clearance experiment, dog B received only small infusions of creatinine except when acute alkalosis was produced by the intravenous administration of large volumes of 0.15 N $NaHCO_3$. Since this introduced a possibly undesirable variation in technique, the clearance studies on the second dog (W) were all carried out with infusions of similar volume but of different composition. The response to the mercurial was qualitatively the same in both dogs; the greater chloruretic effect in dog W is perhaps related to the increase in extracellular fluid volume which resulted from the infusion (*cf.* 24).

The effect of alterations in acid-base metabolism on the response to meralluride was the same in both dogs. When an acute alkalosis was produced during the period of the normal diet, the animal became refractory to the mercurial. In contrast, a prompt diuresis of sodium, chloride and water was obtained when meralluride was administered during chronic hypokalemic alkalosis.[4] In the two courses of potassium depletion in dog B and in the single one in dog W, there was a significant chloruresis each of the eight times that meralluride was given after the animals had become alkalotic, *i.e.*, when the plasma bicarbonate was higher than 30 mEq. per L. and the plasma chloride less than 105 mEq. per L. The data of Figures 1 and 2 suggest that, despite the hypochloremia, the diuretic effect of the mercurial was greater and appeared sooner during the stage of potassium depletion than on the control diet. After the po-

[4] It is recognized that the term hypokalemic alkalosis is somewhat of a misnomer since it defines the disturbance in acid-base metabolism in terms of the plasma level of potassium. Many studies have shown that the persistent extracellular alkalosis is primarily the result of the intracellular potassium deficit. In the present experiments, during the acute alkalosis resulting from sodium bicarbonate infusions, there was a fall in plasma potassium to about the same levels as seen during chronic potassium depletion. These experiments illustrate the failure of the plasma potassium level to serve as an accurate index of the intracellular balance.

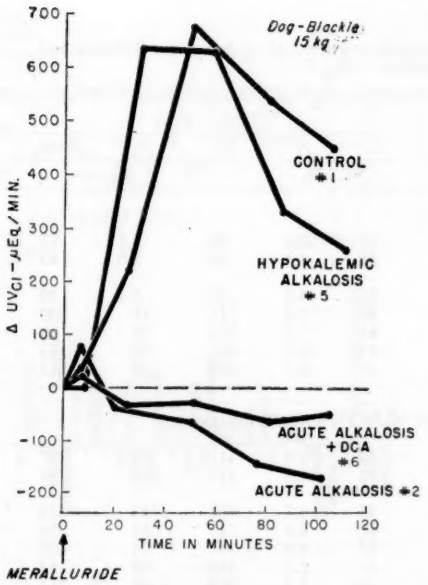

FIG. 1. EFFECT OF MERALLURIDE ON CHLORIDE EXCRETION—DOG B

Mid-point of each collection period is indicated. Change in chloride excretion (ΔUVCl) is corrected for the rate observed during the two control periods before meralluride. For members identifying individual experiments, see Table I.

tassium balance had been restored towards normal and while the administration of DCA was continued, the production of an acute alkalosis was again associated with refractoriness to the mercurial, *cf.* Exps. 6 and 11 of Tables II and III.[5] This indicates that the steroid was not directly responsible for the effectiveness of the mercurial during the period of potassium depletion.

The experiments were designed in an attempt to achieve the same levels of plasma bicarbonate and chloride during acute alkalosis as were obtained during the period of alkalosis due to potassium depletion. As indicated in the tables, the plasma levels were similar under these two conditions, and the marked difference in the chloruretic response cannot be attributed to minor differences in plasma electrolyte concentrations. Indeed, the maximal chloruresis was obtained with the lowest plasma chloride (Exp. No. 10). Similarly, there were only minor fluctuations in the glomerular filtration rate and they bore no relationship to the effectiveness of the diuretic. A possible exception to this is seen in Experiment No. 9, in which the mercurial was given after an acute alkalosis had been superimposed on the alkalosis due to potassium depletion. There was no chloruresis, and this might be attributed either to the lowered filtration rate or to the acute alkalosis *per se*. In experiment No. 10, the filtration rate fell immediately after the period of peak urine flow (*cf.* Table III). This probably represents a hemodynamic response to the sudden dehydration caused by the exceptionally large diuresis.

The results of the second experiment on dog B are given in Figure 3 and demonstrate the persistent chloruretic effect of meralluride during the hypochloremia of potassium depletion. Although a transient period of refractoriness may be sug-

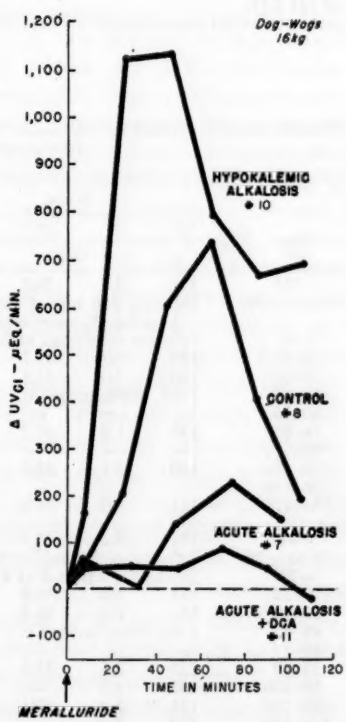

FIG. 2. EFFECT OF MERALLURIDE ON CHLORIDE EXCRETION—DOG W

[5] During acute alkalosis, an increased rate of chloride excretion was noted before the mercury was administered. These changes are similar to those previously described for experiments employing large infusions of sodium bicarbonate (25).

TABLE II

Protocols showing effect of meralluride on electrolyte excretion during alkalosis of potassium depletion and during acute metabolic alkalosis—Dog B

Exp.	Time *min.*	Plasma Na *mEq./L.*	Plasma K *mEq./L.*	Plasma HCO_3 *mEq./L.*	Plasma Cl *mEq./L.*	C_{Cr}† *ml./min.*	V *ml./min.*	Urine Na *μEq./min.*	Urine K *μEq./min.*	Urine HCO_3 *μEq./min.*	Urine Cl *μEq./min.*
5	−46 to −38	Infuse 65 ml. 3.5% creatinine i.v.									
	−38	Continue infusion at 0.5 ml./min.									
	0–24	145	2.5	33.8*	103	39	2.6	30	2.3	25	35
	24–44	151	2.5	33.0*	106	52	4.2	54	5.0	40	45
	46–50	1 ml. Meralluride i.v.									
	44–60	—	—	—	—	38	3.3	51	6.3	30	41
	60–88	149	2.3	32.8	104	50	9.2	771	28	200	678
	88–118	147	2.2	33.0	107	44	7.7	704	39	165	674
	118–142	149	2.5	32.3	103	42	6.2	407	33	153	377
	142–167	147	2.7	—	103	43	5.3	359	29	142	304
6	−70	144	4.3	23.6	112	—	—	—	—	—	—
	−64 to −33	Intravenous infusion of 425 ml. 0.15 N $NaHCO_3$ and 1% creatinine.									
	−33	Infusion continued at 2 ml./min.									
	0–18	148	2.7	33.8	103	50	4.9	317	99	291	141
	18–40	147	2.9	33.9	103	52	4.2	277	108	287	118
	40–45	1 ml. Meralluride i.v.									
	40–55	—	—	—	—	47	4.7	394	112	378	152
	55–76	150	2.8	32.8	107	51	4.4	342	85	350	98
	76–106	151	2.8	33.6	110	47	4.1	361	71	405	101
	106–136	152	2.9	33.8	106	43	2.3	246	69	253	68
	136–154	152	2.9	33.6	103	54	2.3	245	68	252	81

* Blood pH 7.47.
† All urines collected anaerobically without bladder irrigation.

TABLE III

Protocols showing effect of meralluride on electrolyte excretion during alkalosis of potassium depletion and during acute metabolic alkalosis—Dog W

Exp.	Time *min.*	Plasma Na *mEq./L.*	Plasma K *mEq./L.*	Plasma HCO_3 *mEq./L.*	Plasma Cl *mEq./L.*	C_{Cr} *ml./min.*	V *ml./min.*	Urine Na *μEq./min.*	Urine K *μEq./min.*	Urine HCO_3 *μEq./min.*	Urine Cl *μEq./min.*
10	−135	137	2.1	29.5	92.5	—	—	—	—	—	—
	−70 to −30	Infusion 550 ml. of solution containing: NaCl, 90 mEq./L.; KCl, 10 mEq./L.; $NaHCO_3$, 31 mEq./L.; and creatinine, 0.6 per cent.									
	−30	Infusion continued at 3.7 ml./min.									
	0–30	139	1.9	31.7	95.6	60	2.6	166	15	30	110
	30–60	140	2.0	32.5	94.5	57	2.0	160	11	24	106
	60–65	1 ml. Meralluride i.v.									
	60–76	—	—	—	—	60	4.1	359	15	64	269
	76–96	138	1.8	31.7	95.8	56	13.7	1,250	20	51	1,250
	96–116	—	—	—	94.2	52	11.4	1,195	21	29	1,240
	116–136	140	2.1	33.4	92.6	45	7.8	874	23	18	905
	136–156	—	—	—	—	44	6.3	742	22	28	778
	156–176	141	1.9	34.7	90.8	47	6.2	765	22	28	792
11	−84	151	3.2	24.9	110.	—	—	—	—	—	—
	−74 to −26	Infusion of 500 ml. of 0.150 M $NaHCO_3$ containing 0.6 per cent creatinine.									
	−26	Infusion continued at 4.0 ml./min.									
	0–22	151	2.6	35.5	101.	60	4.7	632	60	437	210
	22–40	153	2.5	36.3	101.	58	4.1	559	65	421	162
	40–45	1 ml. Meralluride i.v.									
	40–57	—	—	—	—	60	5.4	775	70	565	229
	57–80	153	2.5	35.8	100.	59	5.3	795	68	538	234
	80–100	152	2.2	—	99.4	61	4.6	730	60	512	230
	100–120	151	2.2	36.1	97.9	62	4.7	776	61	513	272
	120–140	150	2.4	—	97.0	64	4.2	722	56	495	227
	140–160	150	2.4	36.6	96.4	62	3.6	643	51	476	164

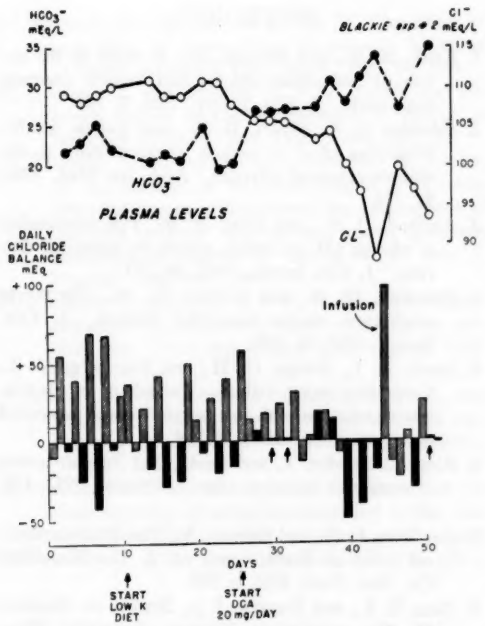

FIG. 3. EFFECT OF REPEATED INJECTIONS OF MERALLURIDE ON CHLORIDE EXCRETION DURING DEVELOPMENT OF ALKALOSIS DUE TO POTASSIUM DEPLETION—DOG B

Meralluride was given intravenously on each day indicated by solid black bars or by arrows. The chloride intake was constant at 84 mEq. per day until day 38, after which all food was refused except for small amounts on days 49 to 51. Infusion on day 45 contained 100 mEq. NaCl and 50 mEq. $NaHCO_3$ in one liter, and was given because of inadequate electrolyte intake. The cumulative balance from day 11 to 52, while on K poor diet, was: K, − 302; Na, + 625; and Cl, + 249 mEq.

As depicted by daily balances, the effect of meralluride on chloride excretion on days 49 and 51 is partially masked by coincidental fluctuations in intake. The output on these days was 46 and 23 mEq. per day, respectively, while on adjacent control days chloride excretion averaged 2.1 mEq. per day.

gested by the daily chloride balance on days 36 and 38, it is equally possible that an early chloruresis was masked by a compensatory conservation of chloride during the latter part of the 24-hour collection period. Mercury increased chloride excretion more at the start of the study when dietary intake was normal, than towards the end, when all food was refused. At this time the animal lost about 150 mEq. of sodium and chloride during one week. A significant decrease in the volume of extracellular fluid and the rate of glomerular filtration may be reasonably inferred. Urinary chloride excretion fell to less than 2 mEq. per day without mercury, but was increased to between 18 and 50 mEq. per day when mercury was administered. An infusion was given on day 45 to restore extracellular volume, and the effect of mercury on subsequent days appeared to have been augmented. This entire experiment illustrates the dependence of mercurial chloruresis on available extracellular fluid. However, despite the severe hypochloremia and the apparent dehydration, the dog did not become refractory to meralluride at any time.

DISCUSSION

The findings clearly indicate that the chloruretic action of mercurials is dependent on factors other than the amount of chloride filtered at the glomerulus, and are consistent with the concept that tubular pH may play an important role. Since potassium depletion is characterized by both intracellular acidosis and the excretion of an acid urine, it is not possible to determine definitely whether it is the pH of the tubular urine or that of the renal cells which conditions the action of the mercurials. Our tentative hypothesis is that the interaction of a cellular constituent and the mercurial may be sensitive to changes in pH. Recently, Benesch and Benesch (26) have reported that reactions between organic mercurials and dithiols are markedly influenced by hydrogen ion concentration. Although there is no direct evidence to implicate cellular dithiols as essential factors in electrolyte transport, the general relationships which have been cited strongly suggest that the pH of the renal cells may affect mercurial diuresis through such a mechanism.

While the present studies emphasize tubular factors, they in no way rule out the possibility that, under many conditions, the filtered chloride load may be the major determinant of mercurial action. Since these agents act by decreasing the reabsorption of the filtered chloride, it is not surprising that their overall effect may be determined by both glomerular and tubular factors.

It has previously been reported that the administration of either DCA (27) or adrenocorticotrophic hormone (28) decreased the diuretic

effect of mercurials. However, a subsequent report by Farah (29) failed to demonstrate any antagonism between DCA and mersalyl. In the present studies DCA had no apparent effect on the action of meralluride. The same degree of chloruresis was noted both before and after the administration of the steroid (*cf.* Exps. 8 and 12). Conversely, DCA did not modify the refractoriness produced by acute metabolic alkalosis.

Many clinical reports have documented the refractoriness to mercurials which appears during alkalosis and which is corrected by the administration of ammonium chloride. However, a survey of the literature has revealed the report of only one patient in whom mercurials retained their effectiveness during severe extracellular alkalosis. Patient S. S. of Schwartz and Wallace (4) responded to meralluride when the serum bicarbonate had risen to 44 mEq. per L. and chloride fallen to 82 mEq. per L. Although the patient was in negative potassium balance, the authors were unable to implicate this as a factor, since other subjects in their series with comparable potassium deficits did become refractory when they developed mild hypochloremia. Further studies are needed before the role of tubular acidosis can be accurately evaluated as a factor in determining the response of patients to mercurial diuretics.

SUMMARY

The diuretic response to organic mercurials was determined in the dog during acute metabolic alkalosis and during chronic alkalosis due to potassium depletion. Acute alkalosis was produced by sodium bicarbonate infusions and was associated with refractoriness to mercurials. When extracellular alkalosis of a similar degree was produced by potassium depletion, the diuretic response to the mercurials was either normal or possibly increased. The results indicate that factors other than the filtered chloride load determine the effectiveness of mercurial diuretics. Since extracellular alkalosis of potassium depletion is accompanied by an intracellular acidosis, it is postulated that increased acidity of the cells of the renal tubule facilitates the interaction between mercurials and cellular components of the electrolyte transport mechanism.

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A STUDY OF THE RATE OF PROTEIN SYNTHESIS BEFORE AND DURING THE ADMINISTRATION OF L-TRIIODOTHYRONINE TO PATIENTS WITH MYXEDEMA AND HEALTHY VOLUNTEERS USING N-15 GLYCINE[1]

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(Submitted for publication August 1, 1955; accepted October 17, 1955)

The purpose of this paper is to report studies of the protein synthesis rate and the size of the metabolic pool using the amino acid, glycine labeled with isotopic nitrogen N-15. Employing the technique described by San Pietro and Rittenberg (1) studies were made on six patients with primary myxedema, four of them before and during the administration of l-triiodothyronine.[2] Five healthy volunteers, one of whom received l-triiodothyronine, are also included in this report.

The N-15 glycine method used in this study is based upon the assumptions that dietary amino acids interact with tissue constituents at a rapid rate (2) and that a dietary amino acid either is used for protein synthesis or is oxidized and its nitrogen excreted.

Figure 1, modified from San Pietro and Rittenberg, illustrates the technique. The metabolic pool (P) is defined as the mixture of nitrogenous compounds derived from the diet and from the breakdown of tissues. Dietary nitrogen enters this pool where part of it is used for protein synthesis. Another part is converted to urea, mixes with the urea already present and is excreted in the urine. The small amount of non-urea nitrogen can be disregarded. The total urea in the body constitutes the urea pool. For the steady

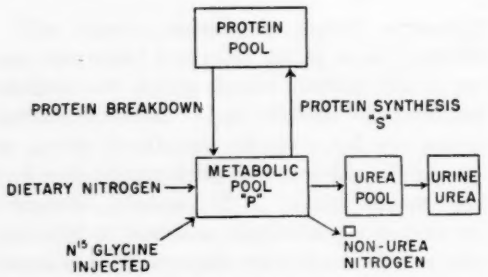

FIG. 1. MODIFIED FROM SAN PIETRO AND RITTENBERG GIVING A SCHEMATIC DIAGRAM OF THE N-15 GLYCINE METHOD

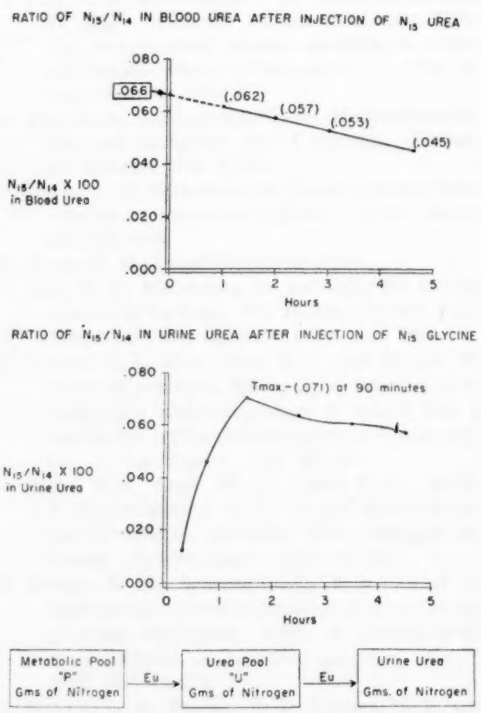

FIG. 2. REPRESENTATIVE CURVES USED TO CALCULATE THE UREA POOL, PROTEIN SYNTHESIS RATE, AND METABOLIC POOL (SEE TEXT)

[1] This study was supported by a grant from the U. S. Public Health Service (C-1815). A preliminary report of this study was given at the International Symposium on Growth Hormone at Henry Ford Hospital, Detroit, Michigan in October, 1954.

[2] Kindly furnished by Dr. Arthur Heming of Smith, Kline, and French Laboratories, Philadelphia, Penna.

TABLE I

Per cent excess of N^{15} urine urea nitrogen

Patient	Therapy	Elapsed time (*minutes*) after injection of N^{15} glycine												
Myxedema		15	20	30	40	45	60	75	90	105	120	150	180	240
R. P.	None		.006		.018		.034	.043	.054	.058	.061	.070	.063	.063
♂	105 Gamma T3 daily—10 days		.015		.037		.057	.070	.073	.072	.072	.068		
	35 Gamma T3 daily—6 months	.011		.028			.081	.054	.061	.047	.064	.066	.066	.064
	35 Gamma T3 daily—14 months													
	70 Gamma T3 daily—1 month		.021		.041		.061	.066	.067		.067		.065	
E. V.	None		.047					.061		.075	.078		.079	.079
♀	105 Gamma T3 daily—10 days					.138	.121	.114	.116			.114	.111	
	53 Gamma T3 daily—2 months	.035		.094		.141	.144	.140	.130	.134	.132	.131	.130	.122
	53 Gamma T3 daily—7 months													
	70 Gamma T3 daily—1 month		.012		.040		.043	.045	.043		.044		.043	
H. P.	None				.009				.033	.044	.048		.051	.053
♀	105 Gamma T3 daily—10 days		.010		.035		.053	.054	.061	.064	.066		.065	.067
B. W.	None	.020									.079	.059	.074	.073
♀	35 Gamma T3 daily—5 days	.020		.045			.114	.095	.095	.087			.090	.093
J. S.	None				.021		.037		.043	.047	.046		.051	.052
♀														
V. G.	None		.020		.054		.073	.076	.080		.083		.085	.067
♀														
Normal														
G. H.	None		.012			.053			.071		.064			.061
♂														
L. G.	None		.013			.066			.148		.120			.112
♂														
L. T.	None		.003				.034	.039	.041	.039	.039		.040	
♂														
S. A.	None				.038			.054	.053	.052	.053	.054	.050	.044
♂	105 Gamma T3 daily—10 days	.095		.097		.090	.144	.086	.065	.063	.063	.066	.066	.064
S. H.	None		.006		.039		.073	.065	.078	.088	.084		.079	.077
♂														

state, dietary nitrogen equals nitrogen excretion and the rate of protein breakdown equals the rate of protein synthesis.

The small amount of the N-15 labeled amino acid glycine which is introduced into the metabolic pool at time zero will be used for protein synthesis or oxidized to urea and excreted. The relative distribution of the N-15 between the protein and urea pools will be a function of the rate of urea excretion and the rate of protein synthesis. If one excretory pathway, *e.g.*, urea (Figure 2), can be completely defined, then it is possible to calculate the rate of protein synthesis and the size of the metabolic pool. This calculation is based upon the assumption that the total transfer of nitrogen from any pool is proportional to the size of that pool. Since the rates of protein synthesis are different in various organs, it is emphasized that this protein synthesis rate is taken as the algebraic sum of all the synthetic rates for the whole organism.

MATERIALS AND METHODS

All subjects were maintained on a constant diet containing approximately 1 Gm. of protein per kilogram of body weight. The nitrogen balance was estimated by analysis of an aliquot of a 24-hour urine specimen and a six-day stool specimen by the macro-Kjeldahl technique (3). An analysis of an aliquot of the diet was made at least once for each study. The patients were all studied while on a metabolic unit and the healthy volunteers carried on their customary activities but were fed on the metabolic unit.

The total body urea was determined by the method of San Pietro and Rittenberg (1) using urea labeled with isotopic nitrogen N-15 (16.0 per cent atom excess) (Figure 2). Approximately three days after determining the total body urea, the subject, while in the fasting state, was given 400 mg. of N-15 glycine (32.0 per cent atom excess) intravenously at 8:00 a.m. One healthy volunteer (S.H.) was given the glycine orally. The urine was then collected over the next four hours at approximately twenty-minute intervals, and the remainder collected to complete a twenty-four-hour specimen (Table I).

An aliquot of each urine specimen was prepared for the mass spectrometer by the method described by

TABLE II

Daily nitrogen balance—GMS

Patient	Before therapy						During therapy							During therapy							
Myxedema							105 Gamma T3 daily—10 days							35 Gamma T3 daily—6 months							
R. P. ♂	+0.6	+2.0	+0.9	+1.2	+0.7	+1.0	+2.2	+1.9	+1.4	+1.8	−1.1	−1.0	−1.2	−2.9	−5.3	−4.5	−5.3	−5.5	−6.9	−6.9	−0.8	−1.0	−0.2	−0.7	−0.6	−0.6	0.0	+0.6
																						35 Gamma T3 daily—16 months; then 70 Gamma T3 daily—6 weeks							
H. P. ♀	+2.2	+1.5	+3.5	+2.1	+1.5	+2.5	+2.3	+3.3	+3.6	+3.6	+3.6	−0.2	−1.4	−0.9	−3.1	−2.5	−2.7	−5.2	−4.4	−3.1	−3.0	−1.3	−0.3	−0.4	−0.5	−1.0	−1.1	−0.4
J. S. ♀	+2.0	+2.9	+2.5	+0.6	+2.1	+1.7	+1.0	+1.6	+2.9	+1.6																		
B. W. ♀				+0.3	+0.9	−1.3	+1.8	−0.1	−0.2	+0.3	0.0	+0.5	35 Gamma T3 daily—10 days −0.9 −2.3 +0.2															
E. V. ♀	+2.2	+2.8	+2.5	+2.2	+1.8	+2.3	+2.1	+1.7	+2.1	+2.2	+2.9	+1.3	105 Gamma T3 daily—10 days −0.8 −1.8 −3.1			−3.2	−3.2	−4.2	−2.6		−1.6	53 Gamma T3 daily—2 months −0.7 −1.2 0.0 −0.9 −0.5 −1.5 −1.2							
																						53 Gamma T3 daily—7 months 70 Gamma T3 daily—1 month							
V. G. ♀	+0.2	+0.6	+0.8	+0.6	+1.0	+1.2	+0.8	+0.4	+1.2	+0.2											−0.2	0.0	−0.1	0.0	−0.7			
Normal																												
S. H. ♂	−0.1	+0.1	−0.1	−0.3	−0.3	+0.2	+0.6	+1.0	+1.4	+0.9																		
L. T. ♂	+1.5	−0.3	−0.1	−0.7	+1.5	+0.6	+0.5	+1.2	+0.3	0.0																		
G. H. ♂	+1.1	+1.1	+1.3	+0.3	+1.6	+2.2	+0.9	+0.1	+0.4	+0.1																		
L. G. ♂	−3.0	−1.9	−2.4	−1.2	−0.4	−0.5	−0.2	+0.1	−0.6	−0.4																		
S. A. ♂	−0.6	+0.4	+0.4	−0.6	+1.2	+0.3	0.0	−1.7	−1.0	−1.1	−1.0	−0.7	105 Gamma T3 daily—10 days −0.7 −1.4 −1.4 −1.2 −0.9 −2.0 −2.0																	

Sprinson and Rittenberg (4). The N-15/N-14 ratio was then determined by means of the mass spectrometer (Consolidated Nier type) with a single slit beam with an accuracy of ± .002 per cent excess.

The studies were carried out in the following subjects:

1. Five studies on five healthy volunteers—L. G., G. H., L. T., and S. A., (♂) and one study on S. A. while receiving l-triiodothyronine 100 micrograms daily over a ten-day period.

2. Fourteen studies on six patients with primary myxedema—R. P. (♂), H. P., E. V., B. W., V. G., and J. S. (♀) in positive nitrogen balance (Table II).

3. Patients R. P., E. V., and H. P. were studied while receiving 100 micrograms of l-triiodothyronine daily for ten days for the first time; and patient B. W. while receiving 35 micrograms daily for the first time.

4. Patient R. P. was restudied after having been on 35 micrograms of l-triiodothyronine for six months and again after receiving 35 micrograms daily for sixteen months and 70 micrograms daily for six weeks. Patient E. V. was restudied after receiving 53 micrograms daily for two months, and again after receiving 53 micrograms daily for seven months and 70 micrograms daily for one month.

RESULTS

The protein synthesis rate, expressed as milligrams of nitrogen per Kg. per 24 hours, was decreased in the patients with myxedema as compared with the healthy volunteers. The average rate for the six patients with myxedema was 354 mg. nitrogen per Kg. per 24 hours (Range 180 to 470 mg. nitrogen) as compared to 756 mg. nitrogen per Kg. per 24 hours (Range 610 to 970 mg. nitrogen) for the healthy volunteers (Table III). The pool size (P) was not consistently altered.

Five of the patients with primary myxedema were in slight to moderate positive nitrogen balance as determined by classical nitrogen balance technique (Table II). The administration of l-triiodothyronine in daily doses of 105 micrograms to patients R. P., E. V., and H. P. resulted in markedly negative nitrogen balance. The protein synthesis rate in patient E. V. measured during this period did not change, while that of H. P. also measured during this period tended to decrease, 490 mg. nitrogen to 318 mg. of nitrogen per Kg. per 24 hours. Patient B. W. received only 35 micrograms of l-triiodothyronine daily for six days and there was no change in nitrogen balance or in the protein synthesis rate during this time.

Patient E. V. was restudied after having re-

TABLE III

Patient	Wt.	Therapy	S	P	U
Myxedema	Kg.	T3	Mg. / Kg. / 24 Hrs.	Mg. / Kg.	Gms.
R. P. ♂	85.2		180	7.0	8.3
	80.1	105 Gamma Daily - 10 Days	370	9.0	7.9
	83.0	35 Gamma Daily - 6 Months	370	4.0	6.6
	80.0	35 Gamma Daily - 14 Months			
		70 Gamma Daily - 1 Month	412	6.5	6.2
E. V. ♀	70.0		350	17.4	5.6
	66.5	105 Gamma Daily - 10 Days	328	4.2	5.0
	66.0	53 Gamma Daily - 2 Months	482	7.0	3.9
	73.0	53 Gamma Daily - 7 Months			
		70 Gamma Daily - 1 Month	1300	22.4	3.9
H. P. ♀	77.0	None	490	21.0	6.1
	74.0	105 Gamma Daily - 10 Days	318	10.0	7.6
B. W. ♀	60.5	None	403	8.6	5.6
	60.0	35 Gamma Daily - 6 Days	400	4.5	5.5
J. S ♀	62.5	None	470	19.0	7.0
V. G. ♀	66.8	None	233	11.5	6.1
Normal					
S. H. ♂	76		670	15.0	4.1
L. T. ♂	81		970	17.0	5.7
G. H. ♂	77.8		850	13.0	5.0
L. G. ♂	77.3		610	12.0	5.0
S. A. ♂	71.0		680	10.0	5.4
	72.0	105 Gamma Daily - 10 Days	126	2.5	6.5

* T-3-L-triiodothyronine, S-protein synthesis rate, P-metabolic pool, U-urea pool.

ceived 53 micrograms of triiodothyronine daily for two months. There was a small increase in synthesis rate, 328 to 482 mg. nitrogen per Kg. per 24 hours. She was still in minimal negative nitrogen balance at this time. After receiving 50 micrograms daily for seven months and 70 micrograms daily for one month the synthesis rate had increased to 1300 mg. nitrogen per Kg. per 24 hours. At this time she was in nitrogen equilibrium.

Patient R. P. was restudied after receiving 35 micrograms of l-triiodothyronine daily for six months. The synthesis rate had increased from 180 to 370 mg. nitrogen per Kg. per 24 hours. After receiving 35 micrograms daily for sixteen months and 70 micrograms daily for six weeks restudy showed a synthesis rate of 412 mg. nitrogen per Kg. per 24 hours. He was in nitrogen equilibrium on the day the last study was carried out.

The administration of 105 micrograms of l-triiodothyronine daily for ten days to a healthy volunteer (S. A.) caused no definite decrease in weight and very minimal negative nitrogen balance but decreased the synthesis rate from a pretreatment level of 680 to 126 mg. nitrogen per Kg. per 24 hours.

DISCUSSION

The protein synthesis rate expressed as milligrams of nitrogen per kilogram per twenty-four hours was consistently decreased in the six patients with primary myxedema (Figure 3, Table III) as compared to the healthy volunteers. This occurred despite the fact that the patients with myxedema were in moderately positive nitrogen balance by classical nitrogen balance studies.

During the first few days that l-triiodothyronine was first administered there was no consistent change in the protein synthesis rate (Table III). In three patients there was little or no change while in one there was an increase toward the normal. The results obtained during this period may not be valid because the steady state has been disturbed and a new one not yet established as manifested by the increasing negative nitrogen balance (Table II).

The continued administration of l-triiodothyronine to two myxedematous patients with the attainment of the euthyroid state and a new steady state as shown by nitrogen equilibrium resulted in a return to a normal protein synthesis rate in one and a marked increase with a return toward normal in the other (Figure 4).

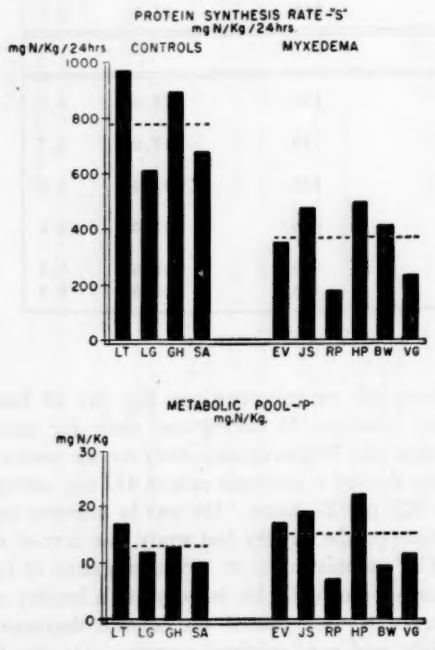

FIG. 3. A COMPARISON OF THE RATE OF PROTEIN SYNTHESIS AND THE SIZE OF THE METABOLIC POOL IN HEALTHY VOLUNTEERS AND PATIENTS WITH MYXEDEMA

The broken horizontal lines represent the average for the various groups.

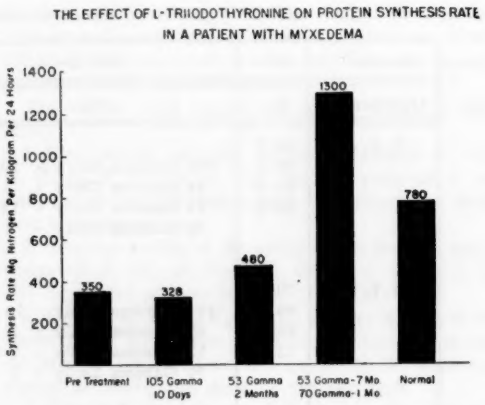

FIG. 4. A COMPARISON OF THE PROTEIN SYNTHESIS RATE OF PATIENT (E. V.), BEFORE AND AFTER THERAPY WITH L-TRIIODOTHYRONINE, WITH THE HEALTHY VOLUNTEERS

DuToit (5) has obtained results *in vitro* similar to these reported here by studying the effect of thyroid hormone on the incorporation of the amino acid alanine into the proteins of rat liver slices using alanine tagged with radioactive carbon, C-14. Thyroidectomy markedly reduces the incorporation rate and the administration of thyroxine to the athyreotic animal restores the incorporation rate to normal. He reports no studies of the effect of thyroxine on normal animals.

From the above animal studies and the results we have obtained in patients with myxedema it would appear that the absence of the thyroid hormone results in an overall decrease in protein synthesis which is corrected by the administration of thyroid hormone. By the present technique we are not able to calculate the rate of breakdown of tissue protein. Olesen, Heilskov, and Schønheyder (6) have recently introduced a new mathematical interpretation of data of this kind. By extending the period of observation over 360 hours rather than 24 hours it is possible to calculate, in addition to pool size and protein synthesis rate, the rate of protein breakdown. Data are being collected according to this technique in patients with myxedema as it seems important to try to delineate what is happening in the relatively acute stage when the patient is in profound negative ni-

trogen balance yet the protein synthesis rate showed little change (Table II, Figure 4). This could mean that "myxedematous tissue" is being catabolized and excreted and that there is a lag period before new normal tissue is formed. The present data are only suggestive and need confirmation by the technique of Olesen, Heilskov, and Schønheyder (6).

The profound negative nitrogen balance and decrease in weight (Figure 5) produced by l-triiodothyronine in the patients with myxedema have been previously reported (7). A similar effect has been reported using dessicated thyroid or thyroxin (8, 9). The administration of l-triiodothyronine in a daily dose of 105 micrograms for ten days to a healthy volunteer (S.A.) produced very minimal negative nitrogen balance and no weight loss. However, there was a marked decrease in the protein synthesis rate from 680 mg. nitrogen per Kg. per 24 hours before therapy to 126 mg. nitrogen per Kg. per 24 hours while receiving the drug. From this single study it would appear that an excess of thyroid hormone also decreases the rate of protein synthesis. We are now studying patients with hyperthyroidism to see if they demonstrate this abnormality.

Rupp, Paschkis, and Cantarow (10) have shown that in the thyroidectomized rat physiological doses of thyroxine decrease nitrogen excretion which they interpret as a protein anabolic effect. Large amounts of thyroxine given to the intact rat caused increased nitrogen excretion or a protein catabolic effect. Our results in man presented here are consistent with the above studies in the rat.

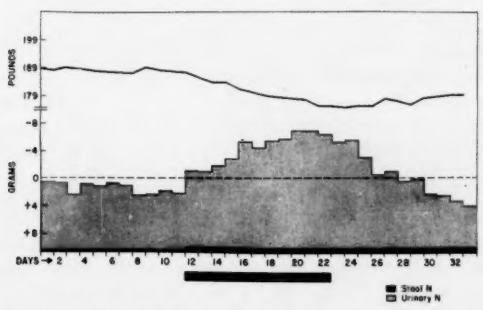

FIG. 5. NITROGEN BALANCE STUDY ON PATIENT R. P.

Results plotted so that below the dotted line is positive nitrogen balance and above the dotted line is negative nitrogen balance.

CONCLUSIONS

1. The protein synthesis rate as measured by the N-15 glycine technique was decreased in six patients with primary myxedema.

2. The administration of l-triiodothyronine restored the protein synthesis rate to a normal level.

3. The administration of triiodothyronine to a healthy volunteer also decreased the rate of protein synthesis suggesting that an excess of thyroid hormone will also decrease the rate of protein synthesis.

4. This N-15 glycine technique appears to offer insight into protein metabolism in man which is not obtainable by classical nitrogen balance studies.

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INSULIN-I^{131} METABOLISM IN HUMAN SUBJECTS: DEMONSTRATION OF INSULIN BINDING GLOBULIN IN THE CIRCULATION OF INSULIN TREATED SUBJECTS [1]

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In recent tracer studies utilizing I^{131} labeled insulin, *in vivo* and *in vitro* metabolic degradation of the labeled insulin has generally been presumed to parallel the disappearance of protein precipitable radioactivity and the appearance of non-precipitable radioactivity (1, 2). The present investigations were prompted by the paucity of data on the metabolic fate of insulin in human subjects and the need for a more specific identification of insulin-I^{131} in blood and tissues than has heretofore been employed. The methods developed during this study have permitted quantitative evaluation of the rate of insulin-I^{131} metabolism and have led to the discovery of an insulin-transporting globulin in the blood of insulin treated subjects.

METHODS

Subjects were patients and healthy volunteer laboratory personnel of the Veterans Administration Hospital, Bronx, N. Y.

Regular crystalline insulin [4] was iodinated by the method of Pressman and Eisen (3) (Method A) or by Newerly's modification of their method as previously described (4) (Method B). Insulin-I^{131} supplied by Abbott Laboratories was also employed in some experiments. The insulin-I^{131} solutions prepared in our laboratory were dialyzed against distilled water with frequent changes for 24 to 72 hours, acidified to pH 3.0 with HCl, maintained sterile by the addition of phenol and cultured before use. These lots of insulin-I^{131} contained an average of 0.1 to 1.0 iodine atoms per molecule, 12,000 molecular weight, insulin. As deduced from the blood sugar responses in fasting rabbits following intravenous administration of labeled and unlabeled insulin, there was no obvious loss in hypoglycemic potency as a result of the manipulations involved in iodination even with preparations containing as many as 2 iodine atoms per molecule insulin of 23,000 M.W., which has been suggested as the functional unit of hormonal activity (5). For most of the *in vivo* experiments presented here, the labeled insulin contained an average of 0.1 to 0.5 iodine atoms per molecule 23,000 M.W. insulin.

The specific activity of the labeled insulin at the time of use ranged from 1.0 μc to 7.5 μc I^{131} per microgram insulin. Doses of 15 to 200 μc containing 0.1 to 7.0 units of insulin were administered intravenously to human subjects and urine collections and heparinized blood samples were taken at intervals thereafter. On the first day of the studies, insulin was withheld from patients receiving daily insulin therapy except in the case of schizophrenic subjects undergoing insulin shock treatment. Assays of radioactivity were carried out on urine, plasma and packed erythrocytes and on the filtrates and washed precipitates of plasma treated with cold 10 per cent trichloracetic acid (TCA) and with 23 per cent sodium sulfate and ether according to the method of Kingsley (6). In many cases radioactivity in the filtrates of the sodium sulfate fractionations was also precipitated with cold 10 per cent TCA after the addition of carrier non-radioactive plasma. By these methods plasma radioactivity was identified as: (a) I^{131} precipitable with TCA, (b) I^{131} not precipitable with TCA, (c) precipitable I^{131} salted out with Na_2SO_4 and ether, and (d) precipitable I^{131} not salted out with Na_2SO_4 and ether. These same procedures were also carried out on control plasma samples to which insulin-I^{131} was added *in vitro*.

Paper electrophoresis was carried out in barbital (pH 8.6) or phosphate (pH 7.3) buffers, $\mu = 0.025$ to 0.10. Strips of Whatman No. 3 MM filter paper, 37 by 550 mm., were stretched horizontally across vertical plastic supports, the ends of the paper dipping down 10 cm. into the buffer vessels. A constant voltage supply was set at levels of 150 to 250 volts. At the end of "runs" lasting 6 to 24 hours, the currents registered were 1 to 2 ma per strip in buffers of 0.025 and 0.05 ionic strength and about 3 ma per strip in buffers of 0.1 ionic strength. After electrophoresis, the strips were stretched horizontally across aluminum pans without contact except at the ends and dry heated in an oven for 45 to 60 minutes at 115° C. After staining with naphthalene black [5] and destaining

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[2] National Heart Institute Research Fellow.

[3] Dazian Foundation Fellow.

[4] We are indebted to Doctors O. K. Behrens and C. W. Pettinga of Eli Lilly Co. for a generous supply of crystalline regular insulin lot No. 535664 which is stated to contain 27 units per mg. This lot served as the source of all the I^{131} labeled insulin prepared in our laboratory.

[5] We are indebted to the Imperial Chemical Industries, Ltd., Manchester, England for a generous supply of this dye.

with 10 per cent acetic acid in methyl alcohol, the plasma protein patterns were traced and the strips divided into 0.5 cm. or 1.0 cm. segments for assay of radioactivity. In some cases the paper strips were counted before and after staining in an automatic strip counting flow gas counter. The counting rates were recorded on moving paper by an Esterline-Angus recorder synchronized with the strip counter. Further details of the paper electrophoresis experiments are given under Results.

Starch block electrophoresis was performed according to the method of Kunkel and Slater (7) in barbital buffer; pH 8.6, $\mu = 0.05$, constant voltage 250 V. After electrophoresis, the blocks were allowed to dry out partly and were divided into 0.5 cm., 1.0 cm. or 2.0 cm. segments for assay of radioactivity.

Ultracentrifugation experiments [6] were carried out in a Spinco preparatory ultracentrifuge, model L. Duplicate specimens of plasma or serum (each 12 ml.) were centrifuged at 40,000 RPM for 7 to 14 hours. The tubes were then removed gently and successive 1-¼ ml. portions were aspirated from top to bottom for assays of radioactivity and determinations of albumin and globulin concentrations. Nitrogen determinations were performed by the Kjeldahl procedure after fractionation of the serum proteins according to the method of Kingsley (6).

Radioactivities in blood and urine samples and in sectioned paper electrophoretograms were assayed in a 5-ml. capacity well-type scintillation counter with a sensitivity of 1.00×10^6 counts per minute per μc I^{131} above a background of approximately 200 counts per minute. The automatic paper strip counter had a sensitivity of 0.60×10^6 counts per minute per μc I^{131} above a background of 20 counts per minute. Starch block sections were assayed in a 10-ml. capacity well-type scintillation counter with a sensitivity of 0.85×10^6 counts per minute per μc I^{131} above a background of 350 counts per minute.

In vivo counting rates over liver, thigh and kidneys were determined with a scintillation counter or a bismuth-wall Geiger counter connected to a Streeter-Amet printing register which recorded cumulative counts at ½ minute or 1-minute intervals.

To compensate for differences in the volume of distribution of insulin-I^{131} in different sized human subjects, blood concentrations were generally plotted as body $wt_{Kg} \times$ fraction of dose administered/liter plasma.

RESULTS

Because of differences in the behavior of insulin-I^{131} in insulin treated and non-insulin treated subjects, it is convenient to distinguish between "control" subjects and insulin treated subjects. As used here, "control" subjects refer either to normal healthy volunteers or hospitalized patients, diabetic or non-diabetic, who had never received injections of insulin prior to this study.

I. In Vivo Experiments

A. Typical radioactivity concentration—time curves in plasma, RBC and urine following intravenous administration of insulin-I^{131}

1. *Control subjects:* Following intravenous injection of insulin-I^{131} in control subjects the concentration of radioactivity falls rapidly in the plasma and rises in the red blood cells and urine (Figure 1a). Even in the earliest collections, no more than 3 per cent of the urinary radioactivity is TCA precipitable with added carrier protein as contrasted with insulin-I^{131} which, when added with carrier protein to urine *in vitro*, is almost entirely (95 to 100 per cent) TCA precipitable. Plasma radioactivity 2 to 4 minutes after injection is largely precipitable with TCA but very shortly thereafter the non-precipitable radioactivity becomes significant and progressively increases for about an hour. Although the concentrations of TCA precipitable radioactivity fall rapidly to quite low levels, the further disappearance of precipitable radioactivity after about 3 hours proceeds at a much slower rate with a half time ranging from 6 to 24 hours. Non-precipitable radioactivity reaches a peak at the end of about one hour, remains at a plateau for an hour or two, and then declines as a result of urinary excretion and an increasing volume of distribution. The curve of erythrocyte radioactivity parallels closely that of the non-precipitable plasma radioactivity at a concentration of about 55 to 60 per cent that of the latter. Since iodide^{131} also distributes between RBC and plasma in this concentration ratio and since we have observed that most of the non-precipitable I^{131} migrates with the same mobility as iodide^{131} on paper electrophoresis, it is quite probable that plasma non-precipitable radioactivity is largely composed of iodide^{131} which represents the metabolic end product of the radioactive moiety of the labeled hormone. Furthermore, the renal clearance of non-precipitable plasma radioactivity averages about 30 ml. per minute which is in good agreement with the average normal rate of iodide clearance (8). Rough calculations based on the concentrations of non-precipitable plasma radioactivity and of the space of distribution of iodide

[6] We are indebted to Mr. Ted Marcus in the laboratory of Dr. Ludwik Gross for aid in these experiments.

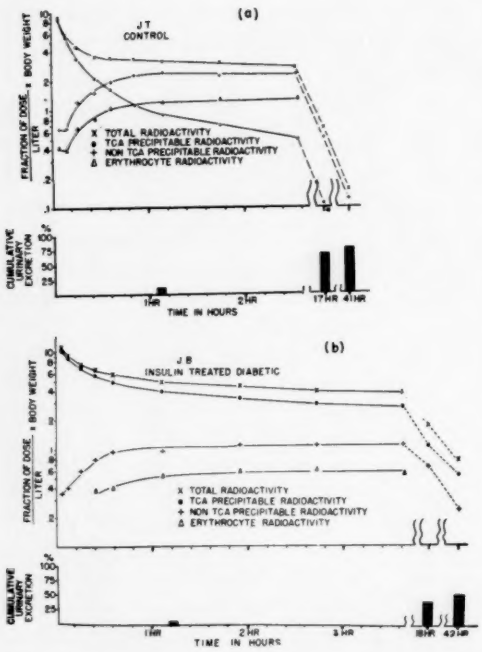

FIG. 1. TYPICAL CURVES OF RADIOACTIVITY IN PLASMA FRACTIONS, ERYTHROCYTES AND URINE AS A FUNCTION OF TIME FOLLOWING INTRAVENOUS ADMINISTRATION OF INSULIN-I^{131}, a) TO SUBJECTS NOT PREVIOUSLY TREATED WITH INSULIN ("CONTROL" SUBJECTS) AND b) TO SUBJECTS TREATED WITH INSULIN FOR MONTHS TO YEARS—BODY WEIGHT IN KILOGRAMS

indicate that from ⅔ to ¾ of the injected insulin-I^{131} is degraded within the first hour.[7]

2. *Insulin treated subjects:* Following insulin-I^{131} administration to patients who have received injections of exogenous insulin for months to years, the general characteristics of the blood radioactivity curves are somewhat similar to those described above. However, the rates of decline of TCA precipitable radioactivity in plasma and of increase in RBC radioactivity and non-precipitable plasma radioactivity are strikingly slower than in control subjects (Figure 1b). Similarly, the early urinary excretions of radioactivity are significantly lower than in control subjects.

The early time courses of TCA precipitable plasma radioactivity are summarized for all subjects in Figure 2. The curves demonstrate a persistence of relatively high concentrations of precipitable radioactivity in the plasma of subjects treated with insulin for more than a few weeks. This group includes 14 patients with diabetes mellitus who had received insulin for 3 months to 17 years, 1 patient (S. P.) with acromegaly and diabetes who had received insulin for 2 years, and 1 schizophrenic patient who had received insulin for 6 weeks. In contrast, a much more rapid disappearance of precipitable radioactivity was observed in 22 non-diabetic control subjects who had never received insulin, 2 diabetic subjects (J. K., J. T.) who had never received insulin, 2 diabetic subjects (M. N., B. B.) who had received insulin for only 2½ and 3½ weeks, respectively, 1 subject (C. B.) with cortisone induced diabetes who had never received insulin and 1 schizophrenic subject (J. Ves.) who had received insulin for 3½ weeks. Subject M. N. showed a rapid disappearance of TCA precipitable radioactivity in the study performed 2½ weeks after insulin therapy was initiated ($M. N._1$) and a slow disappearance in the study performed after 4½ months of insulin therapy ($M. N._2$). Thus, the rate of disappearance of precipitable radioactivity for plasma was correlated only with the history of previous insulin administration and not with the presence or absence of diabetes.

B. Salting out characteristics of TCA precipitable radioactivity in plasma as a function of time following intravenous administration of insulin-I^{131}

When insulin-I^{131} was added *in vitro* to control plasma samples, 90 to 102 per cent of the radioactivity was found in the globulin plaque after the salting out procedure and almost as high a recovery was obtained in venous plasma samples taken 2 to 3 minutes after injection of insulin-I^{131}

[7] *E.g.*, In subject J. T. (weight 66 kg.), the concentration of non-precipitable I^{131} in the plasma at 51 minutes was 3.5 per cent per liter of the total radioactivity administered (Figure 1a). The approximate space of distribution of non-precipitable I^{131} at this time was probably about 17 liters since the space of distribution of iodide^{131}, 30 minutes after intravenous injection, is about 26 per cent of body weight and does not change very much between about 10 minutes and an hour or so (8). To the non-precipitable I^{131} still in the body (17 L. × 3.5 per cent per L. = 59.5 per cent) must be added the 7 per cent excreted in the urine by this time to make a total of 66.5 per cent of the insulin-I^{131} degraded in 51 minutes.

to control subjects. However, in subsequent samples taken from control subjects the fraction of precipitable radioactivity which was salted out by 23 per cent Na_2SO_4 gradually decreased to about 50 per cent by 2 to 3 hours following administration of insulin-I^{131} and persisted at that level without further decrease for another 24 to 30 hours (Figure 3). In contrast with the findings in control subjects, the salting out procedures performed on the plasma of insulin treated subjects almost always revealed a relatively high fraction of the TCA precipitable radioactivity in the globulin plaque even many hours after the administration of the labeled insulin (Figure 3).

C. Analysis of insulin-I^{131} preparations by zone electrophoresis

Since the following observations obtained in barbital or phosphate buffers throughout a range in ionic strength of 0.025 to 0.10, no distinction will be made in the buffer conditions of the various experiments. When tracer amounts of insulin-I^{131} alone or in 10 to 50 microliters of plasma from control subjects are run on Whatman 3 MM paper, the radioactivity is almost quantitatively bound to the paper near the site of application (Figure 4a, b). However, if the paper is soaked in a solution of insulin (3 mg. per ml.) prior to

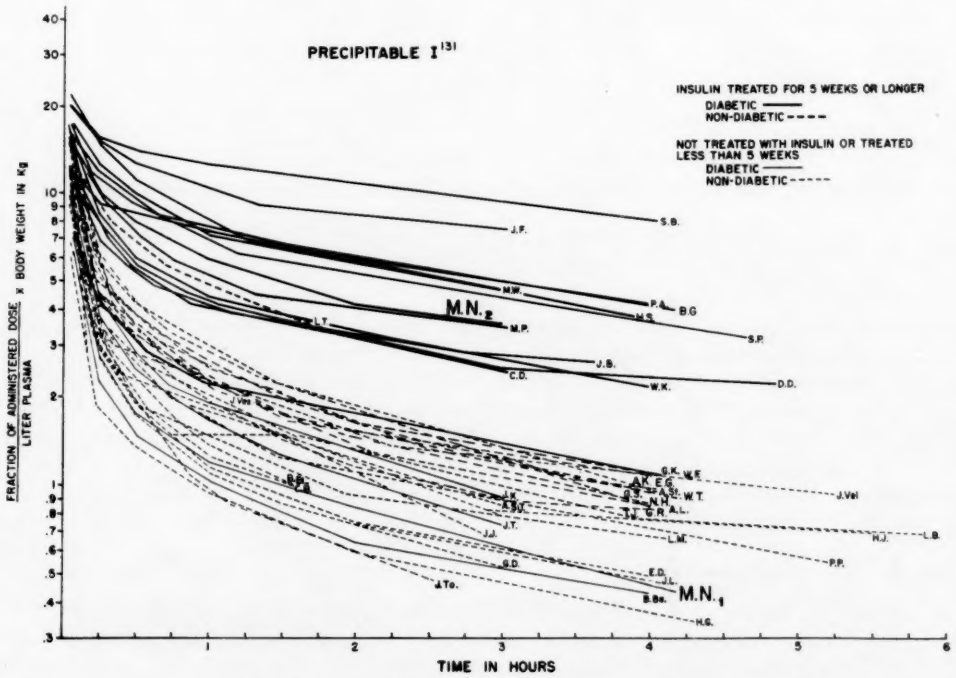

FIG. 2. TRICHLORACETIC ACID PRECIPITABLE RADIOACTIVITY IN PLASMA AS A FUNCTION OF TIME FOLLOWING INTRAVENOUS ADMINISTRATION OF INSULIN-I^{131}

Two studies were performed on subject D. D. In the first of these insulin was withheld for 24 hours prior to intravenous administration of 0.1 unit insulin-I^{131}. In the second study, carried out on the following day, 45 units protamine zinc insulin were given subcutaneously 1 hour prior to the intravenous administration of 7.0 units insulin-I^{131}. The curves were virtually identical and are therefore represented as a single curve. Subject J. Ves. (schizophrenia) and subject L. T. (schizophrenia) received 640 units and 120 units regular insulin, respectively, by subcutaneous injection 45 minutes prior to the intravenous administration of insulin-I^{131}. All other subjects received no stable insulin for at least 24 hours prior to these studies. M. $N._1$—after treatment with insulin for 2½ weeks; M. $N._2$—after treatment with insulin for 4½ months. See text for further identification of subjects.

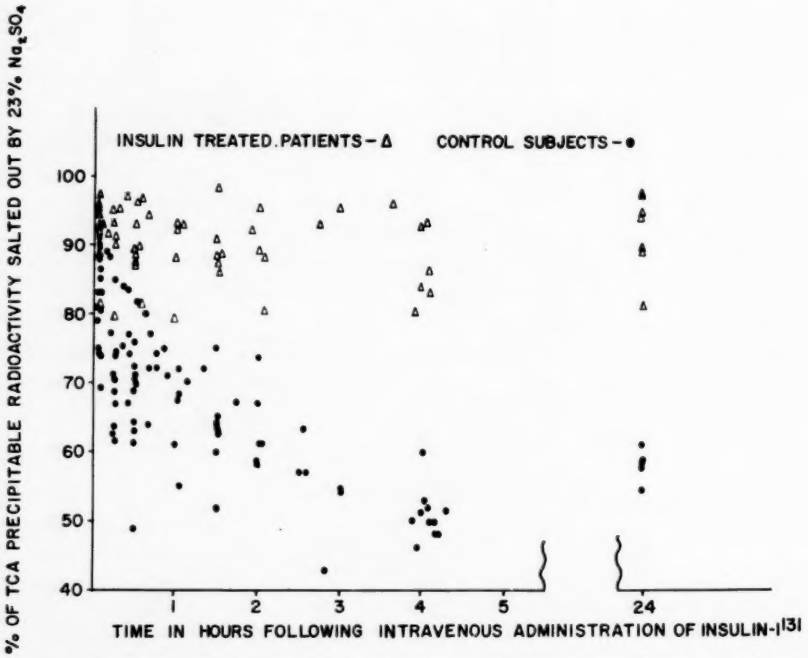

FIG. 3. PER CENT OF TRICHLORACETIC ACID PRECIPITABLE RADIOACTIVITY WHICH WAS SALTED OUT BY 23 PER CENT Na_2SO_4 AS A FUNCTION OF TIME FOLLOWING INTRAVENOUS ADMINISTRATION OF INSULIN-I^{131}

electrophoresis in order to saturate the binding sites, the radioactivity migrates with a mobility somewhat slower than serum albumin whether or not plasma is present (Figure 4c, d). Under the latter conditions the peak of the I^{131} labeled crystalline insulin prepared in our laboratory generally appeared in the α_1 region while in at least one lot prepared by the commercial supplier the

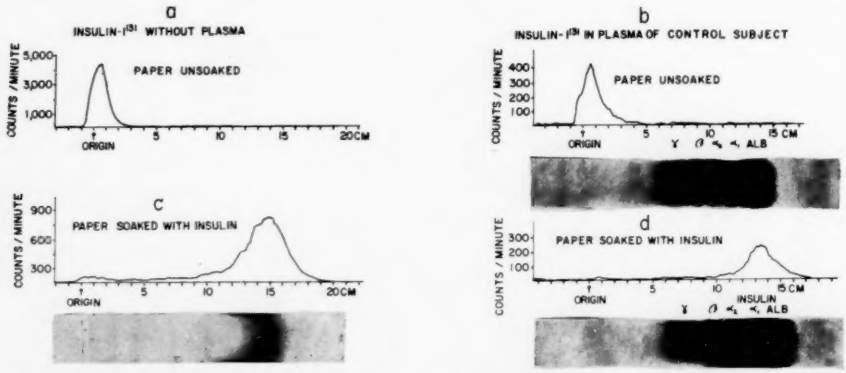

FIG. 4. PAPER RADIOELECTROPHORETOGRAMS: a, c) INSULIN-I^{131} ALONE, b, d) INSULIN-I^{131} IN PLASMA OF A CONTROL SUBJECT

a and b—papers not soaked in insulin prior to electrophoresis; c and d—papers soaked in insulin at origin prior to electrophoresis. Assays of radioactivity by automatic strip counter. The radioactivities which were recorded from the moving paper strip on a curvilinear ordinate scale have been projected on a rectilinear scale.

peak occurred between the β and α_2 proteins. These differences are probably related to variations in the electrophoretic mobility of different native crystalline insulin preparations previously reported by Hall (9) rather than to differences in the methods of iodination. Separation of insulin-I^{131} from the plasma proteins is best demonstrated when the samples containing plasma and insulin-I^{131} are applied along a line close to the cathodal vessel on papers not soaked with insulin and hydrodynamic flow [8] is permitted to move the whole plasma an inch or two towards the anode prior to or during electrophoresis. There is then a protein free zone between the insulin-I^{131} radioactivity bound to the paper at the origin and the slowest migrating γ globulin proteins (Figure 4b).

With all lots of radioiodinated insulin, however, some of the radioactivity migrated with the serum protein fractions. In the most satisfactory preparations only about 2 to 5 per cent of the total activity migrated with the serum proteins, the remainder being adsorbed at the origin (Figure 4b). In some lots, however, as much as 25 per cent of the total radioactivity migrated with the serum proteins which, for reasons indicated below, rendered these preparations much less satisfactory for the study of insulin-I^{131} metabolism.

The chemical nature of the radioactivity accompanying the serum proteins is not clear. Control studies with iodide^{131} added *in vitro* to plasma indicated that less than $\frac{1}{3}$ of 1 per cent of iodide^{131} remains on the paper under the conditions of the experiments reported here, so that the radioactivity migrating with the serum proteins is definitely not due to iodide^{131}, which would be unlikely in any event, since all preparations were dialyzed thoroughly. The possibility that contaminating proteins in the original crystalline insulin are responsible is rendered unlikely by the following experimental observations which indicate that the radioactive material migrating with the serum proteins is somehow produced in the iodination procedures or by other unknown factors. A small quantity of crystalline insulin was divided into two portions. To one portion was added the neutralized iodination mixture used in Method A (3) which contains $NaNO_2$, $KI^{127 \text{ and } 131}$, NaCl and elemental iodine$^{127 \text{ and } 131}$ at a pH of 8.0. From a similar mixture at a pH of about 1.0, elemental iodine was extracted with chloroform according to Method B(4) and added to the other portion of crystalline insulin. At the end of one hour, half of each portion was removed and dialyzed immediately against water for 48 hours. The remainder of each portion was permitted to remain with the iodinating solutions for 48 hours, after which dialysis was carried out in the same manner. All procedures were performed at about 4° C. One ml. of the same plasma was added to a minute amount of each of the four preparations and the mixtures were simultaneously subjected to electrophoresis on paper. In the insulin-I^{131} solutions prepared by iodination for one hour only a small fraction of the total activity migrated with the serum proteins (Figures 5a, b), while the solutions prepared by iodination for 48 hours contained significantly larger fractions of migrating components (Figures 5c, d).[9] These findings were reproducible. Differences in the amounts of iodine bound to the insulin did not appear to be a significant factor. The alterations of insulin-I^{131} which caused a variable fraction to migrate with the serum proteins were not related to the methods of iodination employed since such migrating components were observed in lots prepared by both of the methods used in our laboratory as well as in those produced by the commercial supplier.

D. Electrophoretic behavior of radioactivity in plasma following intravenous administration of insulin-I^{131}

1. Paper electrophoresis: (a) Animal experiments. Since the concentrations of TCA precipitable radioactivity in the plasma of control subjects after about an hour are generally too low to yield quantitatively precise data by paper electrophoresis, two experiments in rabbits receiving

[8] This is effected by leaving open the top of the vapor-saturated enclosure employed for electrophoresis. Under these conditions evaporation of water from the paper strip produces a concentration of buffer salts on the paper thus causing fluid to be drawn up osmotically from the buffer vessels. This produces a rapid movement of the plasma towards the center of the paper strip. If the plasma is applied near the cathodal vessel, hydrodynamic flow moves the plasma in the direction of the anode.

[9] Occasionally, even lots prepared with short iodination periods contained a relatively large fraction of migrating components.

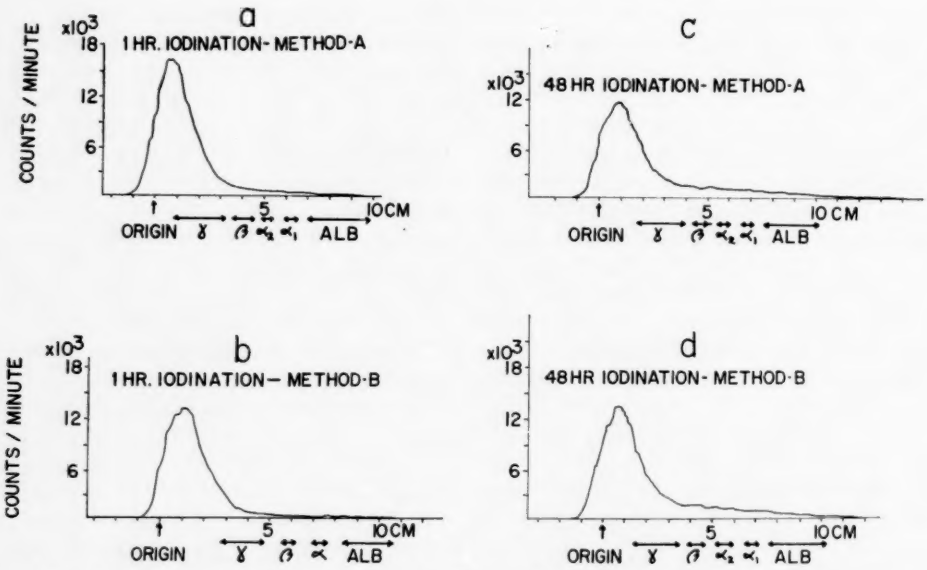

FIG. 5. PAPER RADIOELECTROPHORETOGRAMS OF VARIOUS INSULIN-I^{131} PREPARATIONS IN PLASMA

a) Method A 1-hour iodination
b) Method B 1-hour iodination
c) Method A 48-hour iodination
d) Method B 48-hour iodination

In this and all subsequent figures the paper strips were untreated prior to electrophoresis unless otherwise indicated.

different lots of insulin-I^{131} are shown in Figure 6 for the purpose of illustrating the behavior of the electrophoretic fractions of insulin-I^{131}. Because of the relatively larger amounts of radioactivity administered, high concentrations of TCA precipitable radioactivity were still present at the end of 3 or 4 hours. In rabbit experiment No. 55 insulin-I^{131} lot No. 50 was employed. The radioelectrophoretogram obtained when the labeled insulin was added *in vitro* to a control plasma sample revealed that only about 4 per cent of the total radioactivity migrated with the serum proteins (Figure 6a). Radioelectrophoretograms of plasma samples taken 2 minutes to 3 hours after intravenous administration of this lot of insulin-I^{131} revealed a rapid decrease in the concentration of the unaltered insulin-I^{131} bound to the paper at the origin and a much slower fall in the radioactivity migrating with the serum proteins. Therefore, in later samples, the unaltered insulin-I^{131} gradually became a smaller and smaller fraction of the total radioactivity. The accompanying graph shows that the total number of counts remaining on the paper strips after heat coagulation, staining and destaining was generally in good agreement with the total TCA precipitable radioactivity of the samples. The total counts in the segments corresponding to the unaltered insulin-I^{131}, however, decreased at a much more rapid rate than the originally small moiety of altered insulin migrating with the serum proteins. At the end of one hour, unaltered insulin-I^{131} still comprised over 50 per cent of the total TCA precipitable radioactivity whereas at the 2½-hour point only 15 per cent of the TCA precipitable radioactivity was unaltered insulin-I^{131}.

In experiment No. 59 in which insulin-I^{131} lot No. A611–1674 was employed, the discrepancy between insulin-I^{131} and TCA precipitable radioactivity was much more exaggerated. Approximately 25 per cent of the original radioactivity represented altered products as indicated in the control electrophoretogram (Figure 6b). As early as fifteen minutes after intravenous injection only about 35 per cent of the TCA precipitable radioactivity which remained in the plasma was unaltered insulin-I^{131} and at the end of one hour less than 15 per cent was insulin-I^{131}. This

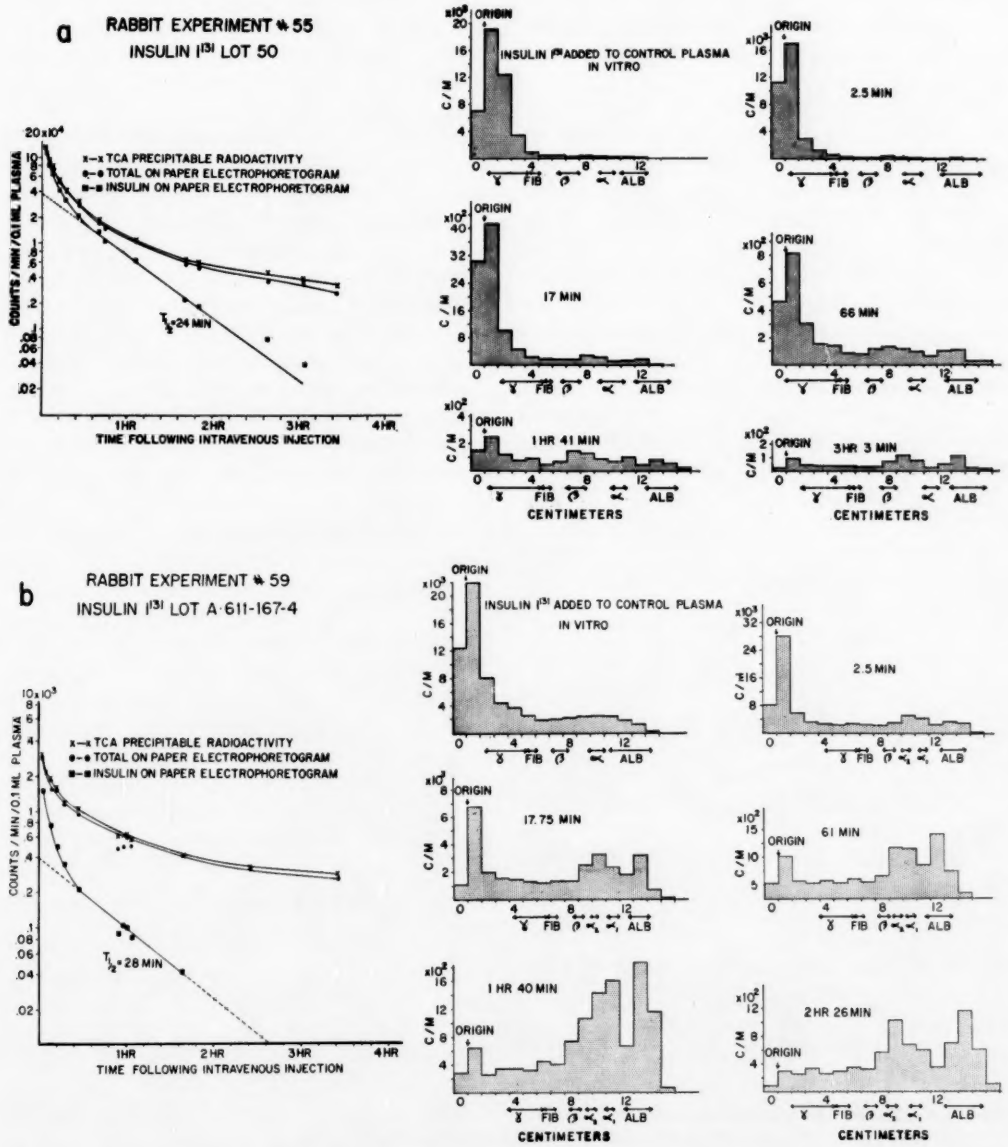

FIG. 6. PAPER RADIOELECTROPHORETOGRAMS OF PLASMA AND CURVES OF TRICHLORACETIC ACID PRECIPITABLE PLASMA RADIOACTIVITY, TOTAL RADIOACTIVITY ON PAPER STRIP AND RADIOACTIVITY ADSORBED TO PAPER AT ORIGIN (INSULIN-I^{131}) AS A FUNCTION OF TIME FOLLOWING INTRAVENOUS ADMINISTRATION OF TWO DIFFERENT LOTS OF INSULIN-I^{131} TO RABBITS

a) Preparation of insulin-I^{131} which contained a small fraction of radioactivity migrating with the plasma proteins.
b) Preparation of insulin-I^{131} which contained a relatively large fraction of radioactivity migrating with plasma proteins.

lot of insulin-I^{131} was also responsible for two of the highest curves of TCA precipitable plasma radioactivity obtained in control human subjects (Figure 2, W. F., G. S.).

The approximate half times for disappearance of unaltered insulin-I^{131}, after the initial distribution phase, were about 25 minutes in both curves.

Electrophoretic patterns similar to those described above have been observed in many other experiments in rabbits and dogs (10), as well as in man, and it may be noted that cross circulation experiments in animals (10) have confirmed that the radioactivity migrating with the serum proteins has a much smaller space of distribution (about 2½ times plasma volume) and a very much slower degradation rate ($T_{1/2}$ equals approximately 18 hours) than that of unaltered insulin-I^{131}.

(*b*) *Control human subjects.* Because of the much lower concentrations of radioactivity obtained in human subjects, plasma samples after the first hour or so are usually not suitable for such electrophoretic analysis. However, in a group of control subjects, 3 to 4 paper strips were run for each plasma sample and the cut sections of each were pooled to yield higher counting rates. This permitted assays of insulin-I^{131} concentrations for periods up to 2 to 3 hours. In several of these cases, a short run of about an hour employing hydrodynamic flow alone without electrophoresis permitted the plasma to be moved through the paper by liquid flow (*i.e.*, simple chromatographic development) and effected a satisfactory separation between insulin-I^{131} adsorbed at the origin and the components migrating with the serum proteins (Figure 7a). The concentrations of un-

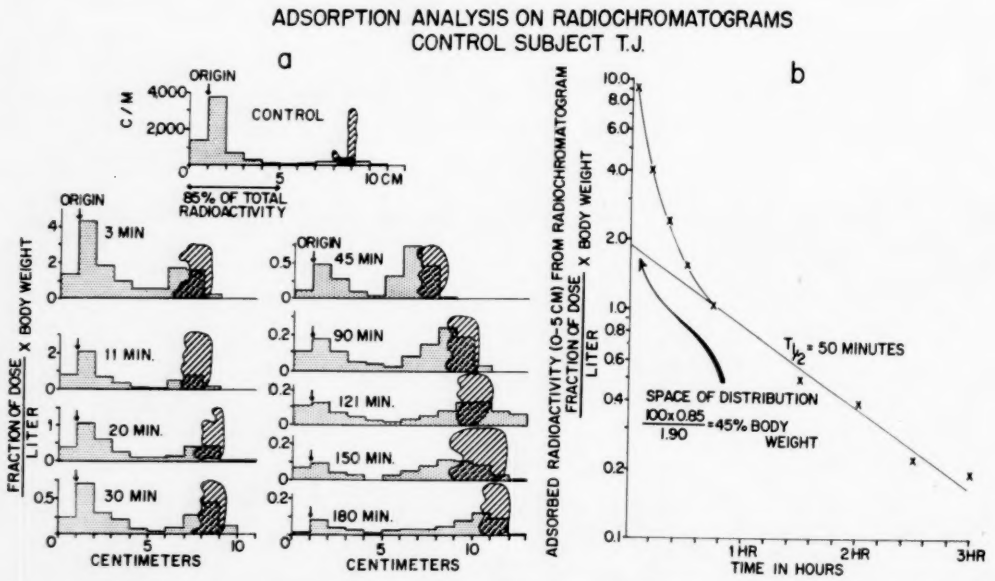

FIG. 7. a) PAPER RADIOCHROMATOGRAMS OF PLASMA AT VARIOUS INTERVALS FOLLOWING INTRAVENOUS ADMINISTRATION OF INSULIN-I^{131} TO CONTROL HUMAN SUBJECT T. J.

Four strips were run for each of the later plasma samples and the cut segments pooled for assay. Bromphenol blue was added to the plasma prior to the runs. The diagonally hatched zones represent the areas of albumin-bound stain.

b) PLASMA CONCENTRATION OF RADIOACTIVITY ADSORBED AT ORIGIN (INSULIN-I^{131}) IN PAPER CHROMATOGRAMS AS A FUNCTION OF TIME IN CONTROL HUMAN SUBJECT T. J.

The early portion of the curve represents distribution and degradation of insulin-I^{131}. The latter exponential segment is presumed to represent degradation primarily or entirely. The zero time extrapolation of the exponential segment gives only an approximate measure of the volume of distribution of the insulin-I^{131}, since it cannot be assumed that the rate of degradation is constant throughout the entire period.

altered insulin-I^{131} as determined from the radioelectrophoretograms or radiochromatograms (Figure 7a), were then plotted as a function of time (Figure 7b). Distribution into an apparent volume averaging about 37 per cent of body weight (Table I) appeared to take place within about 30 to 40 minutes (Figure 7b) following which the plasma concentration fell with an average half time of about 35 minutes (Table I). With some lots of insulin-I^{131}, particularly those containing a relatively large amount of radioactivity migrating with the serum proteins, a decrease in the rate of disappearance from plasma of radioactivity adsorbed at the origin was observed after the concentration had fallen to about 0.2 per cent to 0.4 per cent of the administered dose per liter plasma. It is believed that this small moiety of slowly disappearing radioactivity also represented altered insulin. The length of time required for complete distribution of the labeled insulin in man cannot be precisely determined because of uncertainty regarding the entire course of disappearance from the plasma. However, the exponential decay rates of the electrophoretically determined insulin-I^{131} concentrations are consistent with the rates of metabolic degradation of insulin-I^{131} estimated from the rate of appearance of non-precipitable I^{131} as given above.

(*c*) *Insulin treated subjects.* The radioelectrophoretograms of plasma samples obtained following intravenous administration of insulin-I^{131} to insulin treated subjects differed characteristically from those just described. Plasma samples taken as early at 2 to 3 minutes following injection revealed a significant fraction of the radioactivity migrating with the front running γ globulin ("γ_1" globulins or "β_2" globulins) components of the serum.[10] In some subjects virtually all the radioactivity migrated in this region (Figure 8, S. B.) in all samples taken over several hours while in others there were two distinct peaks, one at the origin representing "free" insulin-I^{131}, the other in the region of the faster migrating γ globulins (Figure 8, G. K.). In some of the latter cases, the patterns obtained in samples taken from the same subject at various intervals after injection were quite similar except for the absolute heights of the peaks, suggesting reestablishment of an equilibrium ratio between the free insulin-I^{131} and the radioactivity migrating with the γ globulins. In other cases, samples taken over the first hour

TABLE I

Space of distribution and rate of degradation of electrophoretically determined insulin-I^{131} in control subjects

Subject	Space of distribution % body weight	Rate of degradation	
		$T_{\frac{1}{2}}$ (min.)	%/min.
A. Su.	38	35	1.97
H. G.	55	42	1.65
J. K.	23	25	2.77
M. N.	39	31	2.23
J. L.	35	30	2.31
B. Ba.	36	38	1.82
N. H.	42	40	1.73
W. T.	48	54	1.28
E. G.	33	43	1.61
W. F.	21	29	2.39
G. S.	23	22	3.16
T. J.	45	50	1.38
Mean	37		2.02

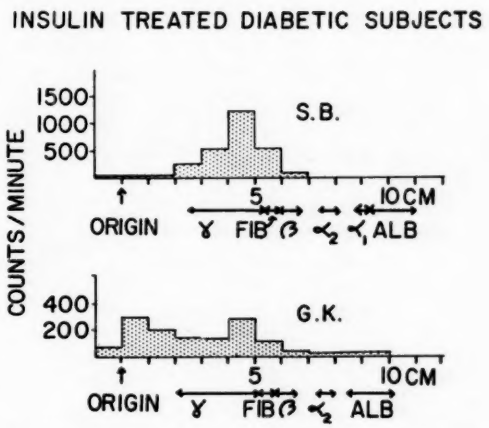

FIG. 8. PAPER RADIOELECTROPHORETOGRAMS OF PLASMA OBTAINED FROM INSULIN-TREATED SUBJECTS 3 MINUTES AFTER INTRAVENOUS ADMINISTRATION OF INSULIN-I^{131}

Subject S. B. Almost all the radioactivity migrated in the region of the front running γ globulin.

Subject G. K. The radioactivity is divided into two peaks, one in the front running γ globulin region, the other near the origin.

[10] Preliminary studies (10) with protein fractions separated with cold ethanol according to the method of Lever and associates (11) suggest that the insulin-I^{131} is complexed to the slowest migrating component in Fraction I and III which contains β_2 and fibrinogen, β_1, α_1 and α_2, rather than to a component of Fraction II which is almost pure γ globulin. Until this is definitely established, however, the site of migration is simply designated as the "front running γ globulin zone."

or so showed two peaks, but samples taken subsequently, when the plasma concentration had decreased significantly, showed only a single peak in the γ globulin region (Figure 9a). This suggested that the earlier concentrations were too high to permit complete binding to the insulin complexing protein. In confirmation of this suggestion the *in vitro* addition of successively larger amounts of labeled insulin to plasma produced a gradual decrease in the fraction bound to the γ globulin even though the absolute amount bound increased (Figure 9b).

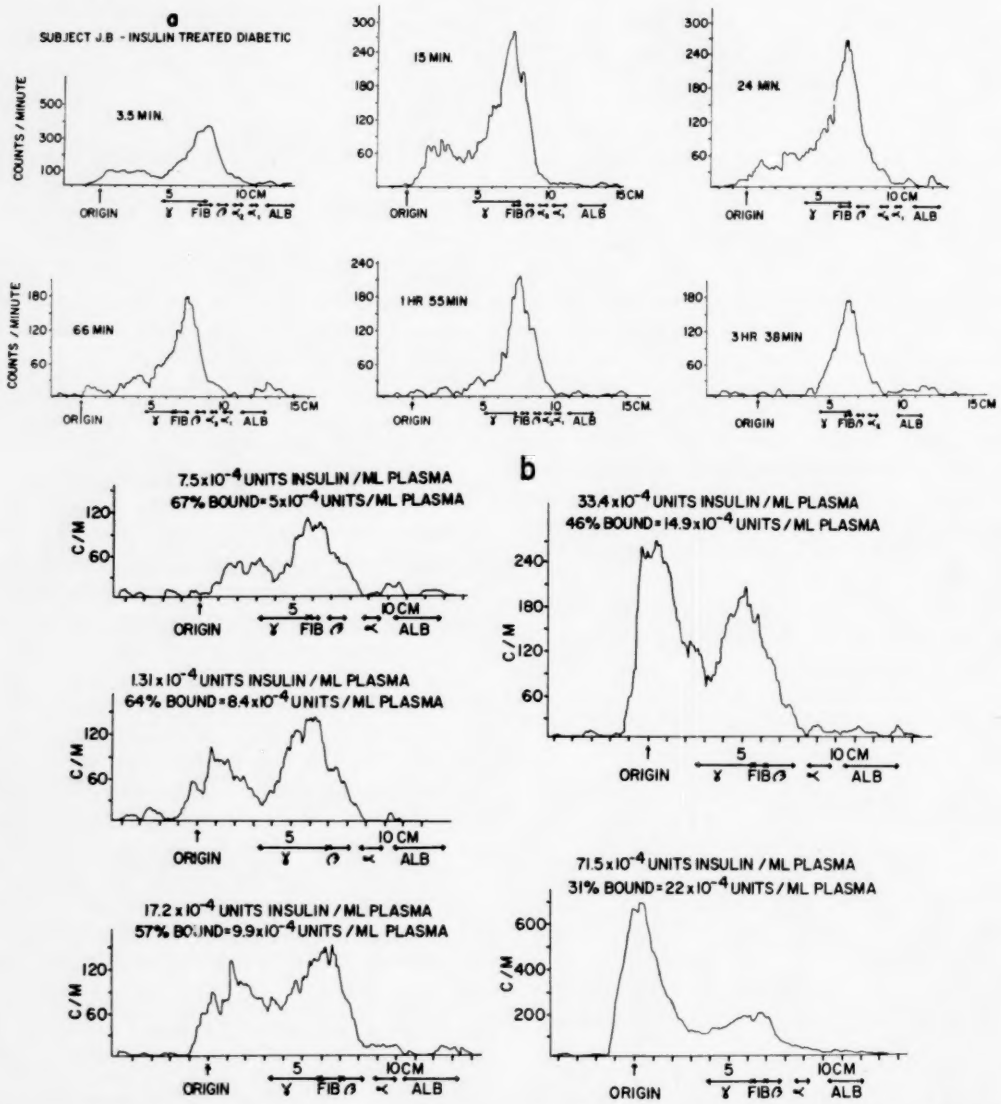
a
SUBJECT J.B - INSULIN TREATED DIABETIC
COUNTS / MINUTE
3.5 MIN.
15 MIN.
24 MIN.
66 MIN
1 HR 55 MIN
3 HR 38 MIN
ORIGIN
FIB
ALB
b
7.5 x 10⁻⁴ UNITS INSULIN / ML PLASMA
67% BOUND = 5 x 10⁻⁴ UNITS / ML PLASMA
1.31 x 10⁻⁴ UNITS INSULIN / ML PLASMA
64% BOUND = 8.4 x 10⁻⁴ UNITS / ML PLASMA
17.2 x 10⁻⁴ UNITS INSULIN / ML PLASMA
57% BOUND = 9.9 x 10⁻⁴ UNITS / ML PLASMA
33.4 x 10⁻⁴ UNITS INSULIN / ML PLASMA
46% BOUND = 14.9 x 10⁻⁴ UNITS / ML PLASMA
71.5 x 10⁻⁴ UNITS INSULIN / ML PLASMA
31% BOUND = 22 x 10⁻⁴ UNITS / ML PLASMA
C/M
10 CM
15 CM

FIG. 9. a) PAPER RADIOELECTROPHORETOGRAMS OF PLASMA SAMPLES TAKEN AT VARIOUS INTERVALS FOLLOWING INTRAVENOUS ADMINISTRATION OF INSULIN-I^{131} TO SUBJECT J. B., A DIABETIC PATIENT TREATED WITH INSULIN FOR 15 YEARS

b) TWO-HOUR PLASMA SAMPLE FROM J. B., TO WHICH VARYING AMOUNTS OF INSULIN-I^{131} WERE ADDED *in Vitro*

Frequently, when the same sample was run under somewhat different conditions, variations in the relative heights of the two peaks was observed. The results of a large number of experiments of this sort indicated quite definitely that dissociation of the insulin-γ globulin complex was taking place during electrophoresis, and that the paper was competing with the γ globulin for the free insulin-I^{131}. Thus, in cases where dissociation of the insulin-γ globulin complex appeared to be relatively rapid, the tendency towards dissociation could be enhanced by permitting the plasma to remain at the origin for a few hours prior to slow electrophoretic migration in high ionic strength buffers. A larger peak at the origin and a considerable degree of trailing towards the front running γ globulin peak were then observed and consequently a smaller peak was obtained in the front running γ globulin region. Conversely, dissociation could be inhibited by producing a rapid hydrodynamic movement of the whole plasma towards the anode prior to electrophoresis, with the consequent maintenance of a high peak in the front running γ globulin zone and a lesser degree of trailing between this region and the free insulin-I^{131} bound at the origin. Because of the competition for insulin-I^{131} between the paper and the γ globulin, the kinetics of association and dissociation of the insulin-γ globulin complex could not be studied satisfactorily by this means.

When plasma samples from these subjects were run on paper strips presoaked in nonradioactive insulin solutions, two peaks were again observed, with the "free" insulin-I^{131} no longer bound to the paper at the origin but running just behind the albumin (Figure 10). Generally, the ratio, $\frac{\gamma \text{ globulin bound insulin-}I^{131}}{\text{"free" insulin-}I^{131}}$ was decreased by this procedure, apparently as a result of exchange between the nonradioactive insulin used in soaking the paper and radioactive insulin in the γ globulin complex and a consequent lowering of the ratio of total γ globulin bound insulin to total free insulin. Even when the insulin presoaked papers were heat coagulated, stained and destained, this exchange could not be entirely prevented. Such displacement of radioactive insulin by nonradioactive insulin could readily be demonstrated even in samples where all the radioactive insulin was originally γ globulin bound prior to the addition of nonradioactive insulin.

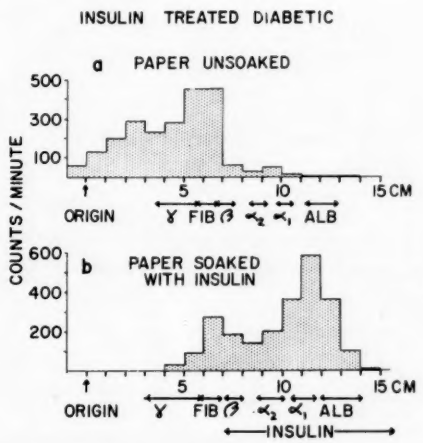

FIG. 10. PAPER RADIOELECTROPHORETOGRAMS OF PLASMA FROM SUBJECT B. G., A DIABETIC PATIENT TREATED WITH INSULIN FOR 10 YEARS

a) Paper not soaked in insulin prior to electrophoresis.
b) Paper soaked in insulin prior to electrophoresis.

There was a very close correlation between the extent of γ globulin binding of insulin and the rate of fall in the precipitable radioactivity of the plasma. Where almost all the radioactivity was present in the γ globulin peak, the plasma concentrations decreased at a very slow rate. Conversely, when the γ globulin peak was smaller than the free insulin peak, the precipitable radioactivity in the plasma declined more rapidly. No definite correlation could be established between the extent of insulin binding to γ globulin or the rate of fall of plasma precipitable radioactivity and the insulin requirements of the patients studied. Furthermore, some patients who had taken insulin for 15 years or longer manifested relatively weak binding of insulin to γ globulin while others with much shorter histories of insulin therapy revealed strong binding. In subject M. N. no evidence of γ globulin binding was obtained after 2½ weeks of insulin therapy (Figure 11a). However, on repeat study 4½ months later there was obvious strong binding to γ globulin (Figure 11b). These findings correlated well with the different rates of disappearance of TCA precipitable radioactivity from the plasma (Figure 2, M. $N._1$ and M. $N._2$).

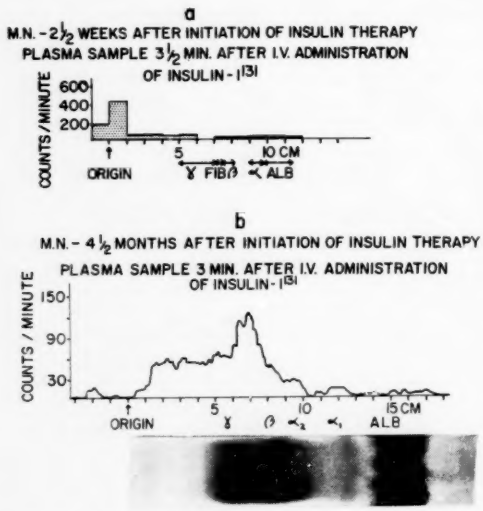

FIG. 11. PAPER RADIOELECTROPHORETOGRAMS OF PLASMA FOLLOWING INTRAVENOUS ADMINISTRATION OF INSULIN-I^{131} TO SUBJECT M. N.

a) After 2½ weeks of insulin therapy.
b) After 4½ months of insulin therapy.

Binding of the insulin-I^{131} to the γ globulin also occurred upon the addition of insulin-I^{131} *in vitro* to the plasma of these subjects (Figure 9b). Other *in vitro* studies are presented below.

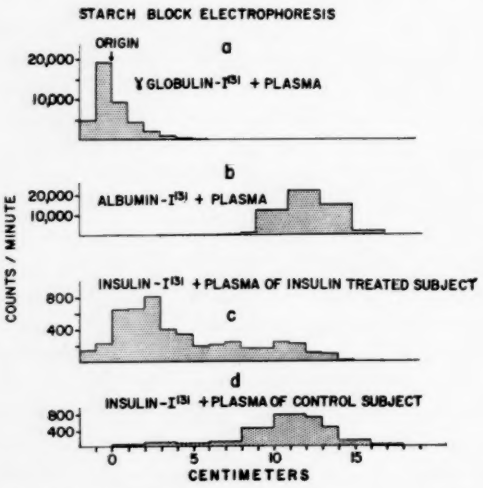

FIG. 12. STARCH BLOCK RADIOELECTROPHORETOGRAMS

The four specimens were run simultaneously on the same block.

2. Starch block electrophoresis: When insulin-I^{131} in the plasma of control subjects is run on the starch block, binding to starch does not occur to any significant extent since there is a single peak of radioactivity just behind that of serum albumin [11] (Figure 12d) and the small extent of trailing which is observed can be explained on the basis of altered components migrating with the serum proteins. In the plasma of insulin treated subjects, however, there is again either a single peak in the fore-running γ globulin zone, or two peaks, one corresponding to the front of the γ globulin region and one in the region of free insulin migration behind the albumin (Figure 12c). At least partial displacement of insulin-I^{131} from the γ

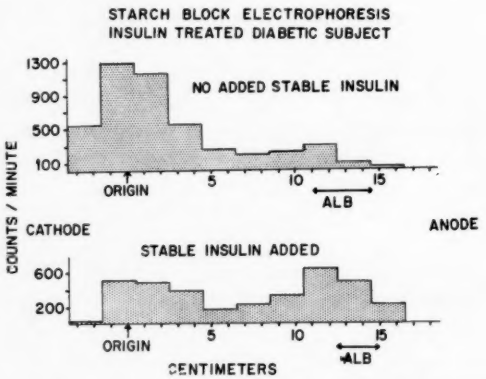

FIG. 13. STARCH BLOCK RADIOELECTROPHORETOGRAMS OF PLASMA FROM SUBJECT J. B. WHO HAD BEEN TREATED WITH INSULIN FOR 15 YEARS

Bromphenol blue was added to the plasma to identify the zone of albumin migration.

Top—without added stable insulin.

Bottom—stable insulin added to plasma 8 hours prior to electrophoresis.

globulin complex to the region of free insulin migration is readily demonstrated by the addition of nonradioactive insulin to the labeled plasma (Figure 13).

E. External assays of radioactivity over liver, kidneys and thigh

Simultaneous counting rates over liver and thigh, or over liver and kidneys, were recorded in

[11] When a small amount of insulin-I^{131} in water is run on the starch block the peak occurs in the same position as when the insulin-I^{131} is run in plasma.

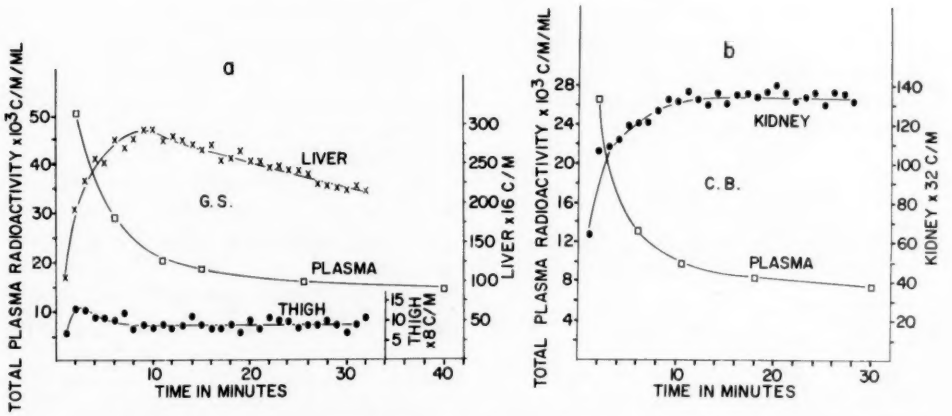

FIG. 14. RADIOACTIVITIES OVER LIVER, THIGH AND KIDNEY COMPARED TO CONCENTRATIONS OF RADIOACTIVITY IN PLASMA AS A FUNCTION OF TIME FOLLOWING INTRAVENOUS ADMINISTRATION OF INSULIN-I^{131} TO CONTROL HUMAN SUBJECTS

several patients (Figure 14). The thigh counts reached a peak within about one-half to 2 minutes after injection and then decreased in a manner roughly parallel to the curve of total plasma radioactivity. Over the liver (Figure 14a), however, there was occasionally recorded a continual buildup to a peak for a period of about 10 minutes following which radioactivity decreased at a somewhat faster rate than over the thigh or in the blood. Increasing radioactivity over the kidneys for about 8 to 10 minutes was also frequently observed (Figure 14b). Although the methods of *in vivo* assay are so rough as to permit conclusions only of a qualitative nature, the increase in radioactivity over liver and kidneys at a time when radioactivity in plasma and over the thigh are de-

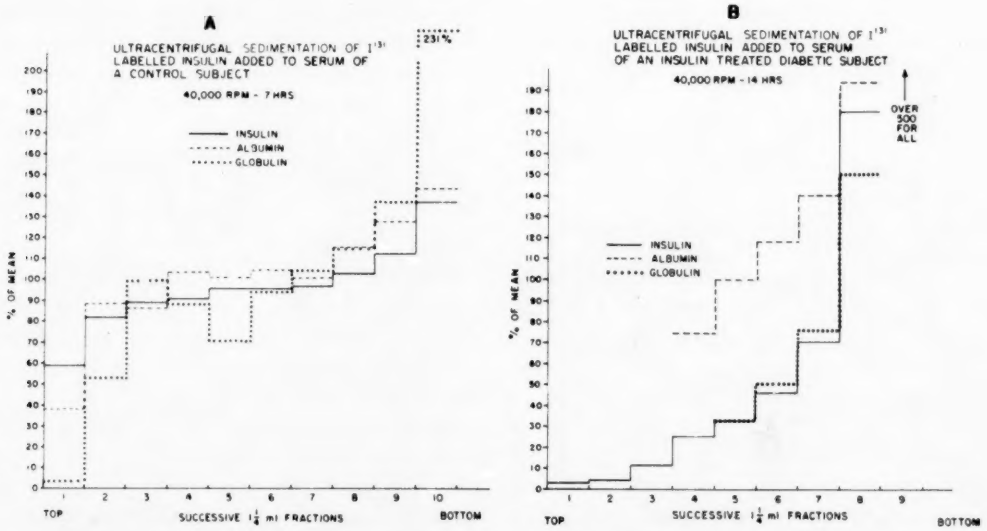

FIG. 15. ANALYSIS AFTER ULTRACENTRIFUGATION OF INSULIN-I^{131} AND SEDIMENTING PROTEIN FRACTIONS

a) Insulin-I^{131} in serum of control subject.

b) Insulin-I^{131} in serum of D. D., a diabetic patient treated with insulin for 13 years.

creasing would seem to indicate a heavy concentration of insulin-I^{131} in these organs, which probably represent sites of insulin degradation. This is in accord with the *in vitro* studies of Elgee, Williams, and Lee (2).

II. In Vitro Studies

A. Ultracentrifugal sedimentation of insulin-I^{131} in the plasma of control and insulin treated subjects

When insulin-I^{131} is added to the plasma of a control subject, the radioactivity sediments less rapidly than serum albumin (Figure 15a) as might be expected from the relatively low molecular weight of insulin. However, when insulin-I^{131} is added to the plasma of an insulin treated subject, the radioactivity sediments with the globulins, indicating a binding of insulin to one of the globulin components (Figure 15b).

B. Effect of in vitro incubation of insulin-I^{131} with plasma

When insulin-I^{131} was incubated with control plasma at 37° C., some non-precipitable radioactivity appeared to the extent of about 30 per cent of the total after 72 hours. A more interesting finding, however, was the gradual disappearance of the free insulin-I^{131} peak on electrophoresis and a buildup of radioactivity in the α_2 globulin region so that at the end of 72 hours much of the precipitable radioactivity was frequently present in this region (Figure 16). In control experiments no detectable changes were observed in the radioelectrophoretograms of I^{131} labeled γ globulin or I^{131} labeled serum albumin after 74 hours' incubation with plasma (Figure 16). Heating of the plasma for one hour at 57° prior to incubation with insulin-I^{131} did not prevent the changes noted above. Incubation of insulin-I^{131} and/or plasma alone for 72 hours did not result in these findings on electrophoresis of the insulin-I^{131} and plasma together. Experiments in dogs showed that the biologic activity of the insulin-I^{131} preparations after 24 hours' incubation was approximately proportional to the free insulin peak remaining. Unlike fresh solutions of insulin-I^{131}, the 72-hour incubated mixtures of insulin-I^{131} and plasma, when injected into dogs, showed a small space of distribution and a slow rate of removal of precipitable radioactivity from the plasma (10).

When insulin-I^{131} was incubated with the plasma of insulin treated subjects who showed strong binding to γ globulin, the amounts of non-precipitable radioactivity which migrated in the α_2 globulin region were much less than in control subjects. Labeled insulin bound to γ globulin appeared to be partially "protected" from degradation in these cases.

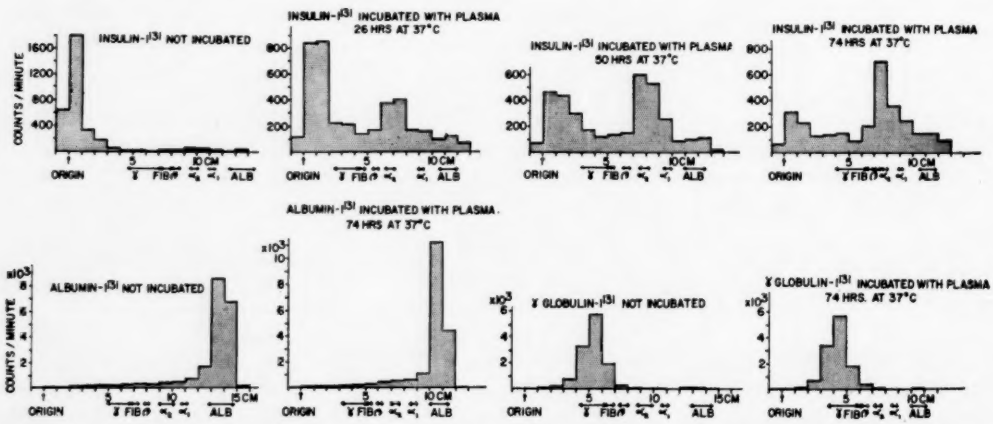
INSULIN-I131 NOT INCUBATED
INSULIN-I131 INCUBATED WITH PLASMA 26 HRS AT 37°C
INSULIN-I131 INCUBATED WITH PLASMA 50 HRS AT 37°C
INSULIN-I131 INCUBATED WITH PLASMA 74 HRS AT 37°C
ALBUMIN-I131 NOT INCUBATED
ALBUMIN-I131 INCUBATED WITH PLASMA 74 HRS AT 37°C
γ GLOBULIN-I131 NOT INCUBATED
γ GLOBULIN-I131 INCUBATED WITH PLASMA 74 HRS. AT 37°C
COUNTS / MINUTE
ORIGIN
γ FIB
ALB

FIG. 16. RADIOELECTROPHORETOGRAMS OF PLASMA INCUBATED WITH INSULIN-I^{131}, ALBUMIN-I^{131} AND γ GLOBULIN-I^{131} FOR VARIOUS PERIODS OF TIME

TABLE II

Binding of insulin-I^{131} to γ globulin in insulin treated and untreated subjects

	Diagnosis	Duration of insulin therapy	Number of subjects
Binding present	Diabetes mellitus	7½ weeks to 23 years	25
	Diabetes with acromegaly	14 months	1
	Schizophrenia	6 weeks, 3½ months	2
Binding absent	Diabetes mellitus	0	5
		8 days–12 weeks	6
	Cortisone induced diabetes	0	1
	Schizophrenia	3½ weeks, 8 weeks	2
	Non-diabetic; nonschizophrenic controls	0	33

C. Electrophoretic analysis of insulin-I^{131} added in vitro to plasma of insulin treated and non-treated subjects

In addition to the patients who received intravenous injections of insulin-I^{131} (Figure 2), plasma or serum samples were obtained from a large number of subjects and subjected to paper and starch block electrophoresis after addition of insulin-I^{131} *in vitro*. Binding of insulin-I^{131} to γ globulin was never observed in the absence of previous insulin therapy but was always present in subjects who had received insulin treatment for 3 months or longer. This latter group included 13 diabetic patients and 1 schizophrenic non-diabetic subject in addition to the schizophrenic subject (W. T.) mentioned above. In another group of diabetic and schizophrenic patients who had received insulin for 12 weeks or less, binding of insulin-I^{131} to γ globulin was not observed. In one diabetic patient treated with insulin for 7½ weeks, insulin binding by γ globulin was observed. Table II summarizes the data for the entire group of 75 subjects.

DISCUSSION

It has previously been observed (12) that subtle alterations of proteins produced by isotopic labeling procedures may escape detection in any of a number of physical, chemical or biological testing systems. Thus, even gross inhomogeneities in I^{131} labeled human serum albumin frequently remained undiscovered by electrophoretic and ultracentrifugal analysis but showed up as more or less rapidly degraded components following injection into man (12). In the case of a protein or peptide hormone such as insulin, any *drastic* changes induced by the labeling process are likely to be revealed by a decrease in biologic activity. However, it is well known that insulin can tolerate certain minor changes, such as substitution on some of the tyrosine residues, without sacrifice of biologic potency (13). Furthermore, while the most sensitive of available procedures for testing of insulin potency may be reliable to within about 5 to 10 per cent, the blood sugar response of fasting rabbits utilized here is probably incapable of detecting loss of potency of the order of 20 to 30 per cent or more. Thus, even if each lot of labeled insulin is subjected to exhaustive biologic testing, which is virtually a prohibitive enterprise, biologic alteration of a small fraction of the starting material cannot be excluded.

The problem of whether the separation of the radioactive label from the protein is synonymous with metabolic degradation of the protein molecule has previously been discussed in detail (12). The fact that the rate of separation of I^{131} from the labeled insulin can be slowed significantly by the simultaneous administration of very large doses of unlabeled insulin ($>$ 200 U per kg. body weight) to rabbits (10) or by the addition of stable insulin, *in vitro*, to liver preparations which degrade insulin (14) suggests at least that the two processes are related and that the degrading mechanisms do not distinguish between the labeled and native insulin. However, as emphasized above, complete integrity of the labeled insulin cannot be guaranteed and the presence of even a small fraction of altered material is capable of introducing very annoying complexities into the experimental studies. When, in addition, the magnitude of the fraction of altered material varies among different preparations of labeled insulin

derived from the same lot of crystalline insulin, the difficulties are multiplied. In the present study the most specific method for the identification of unaltered insulin-I^{131} in plasma was found to be paper electrophoresis. Even this method, however, is not eminently satisfactory since it simply permits distinction between protein bound radioactivity which migrates with the serum proteins (and is therefore definitely not unaltered insulin) from protein bound radioactivity which is adsorbed at the starting line (and may therefore represent unaltered insulin). Yet, it can at least be accepted that most of the adsorbed material is, in fact, unaltered insulin, since it comprises a major fraction of the insulin-I^{131} preparations which show no definite loss in biologic potency.

On the basis of the foregoing discussion it therefore seems reasonable to regard the fall in plasma concentration of the paper adsorbed radioactivity as reflecting the distribution and metabolism of unaltered insulin-I^{131} and to utilize the data obtained in this manner to attempt an estimation of endogenous insulin concentrations in the plasma of normal subjects. Very roughly, and perhaps arbitrarily, the daily utilization of insulin is taken at 48 units, based in part on the insulin requirements of depancreatized subjects. This reduces to a value of 1 unit of insulin metabolized in 30 minutes. Again roughly, the results of the present study indicate that I^{131} labeled insulin distributes into an apparent volume of 26 liters in the average sized subject and is degraded at a rate of about 2.0 per cent per minute. In 30 minutes, therefore, approximately 60 per cent of the insulin in body fluids is turned over. Since this amount represents about 1 unit of insulin, the total volume of distribution, 26 liters, contains about 1/0.60 units. The average insulin concentration is then $\frac{1.7 \text{ units}}{26 \text{ liters}}$ or 0.065 units per liter. Since each unit corresponds to 37 micrograms, this concentration is equivalent to about 2½ micrograms insulin per liter. These calculations should not be construed as implying a constancy of insulin concentration; on the contrary, it is recognized that there are probably marked fluctuations of insulin secretion in response to food intake or physiologic stresses. The values presented are merely rough estimates for an overall time average. That the calculated average value of 1.7 units for the total insulin present in body fluids and readily exchangeable with plasma insulin is at least not an inordinately low estimate is confirmed by observations that as little as twice this amount administered intravenously is capable of producing a definite although transient fall in blood sugar (15).

Since the estimated total exchangeable insulin is only of the order of one and a half units, it may be questioned whether the doses administered in the present study (0.1 to 7.0 units) can be considered as tracer doses in the sense of being physiologically insignificant. Certainly, the larger doses are actually in the pharmacological range while even the smaller doses, though without discernible effect, cannot be considered negligible. However, it should be noted that in one subject (J. Ves.) who had received 640 units regular insulin subcutaneously 40 minutes prior to intravenous injection of the tagged insulin, the rate of disappearance of the tagged insulin did not differ significantly from that of other control subjects (Figure 2). Since this patient developed progressively severe hypoglycemic symptoms during the course of the study, there is little question that much higher than normal insulin concentrations in plasma were obtained. Furthermore, in rabbit experiments it has been found that there is no significant change in the rate of insulin-I^{131} degradation until accompanying doses of stable insulin administered intravenously reach levels as high as 100 units to 200 units per kg. body weight (10). Above this level the rate of insulin-I^{131} degradation decreases significantly, probably as a result of overloading of the insulin degrading mechanisms. A similar decrease in the rate of degradation of I^{131} tagged globulin produced by overloading with large doses of stable globin has previously been reported (16). In the case of insulin, therefore, it appears as if the degradation reaction follows first order kinetics over a very wide range of insulin concentrations. Similarly, Hellman and his associates (17) showed that C^{14} labeled hydrocortisone was conjugated and excreted at the same rate when 0.25 milligrams or 100 milligrams were administered to a patient devoid of endogenous sources of this hormone. These results suggest that at any particular metabolic level the rate constant for inactivation, by degradation or conjugation, of some hormones remains constant over a range of hormone concentrations far exceeding physiologic

limits. In most cases, the rate of inactivation appears to be very rapid (in comparison to the rate of metabolism of serum proteins) with a half time of the order of 30 minutes or so. This is true of insulin, hydrocortisone and several of the anterior pituitary hormones (18, 19). While this pattern of hormone inactivation is undoubtedly responsible for tremendous hormone waste, it allows for temporarily high concentrations in response to physiologic demands or stress situations without the penalty of prolongation of effect after the need has passed.

One notable exception to this scheme is thyroxine which is normally degraded with a half time about 7 days (20) and whose degradation follows second order kinetics (20, 21). The latter behavior is due to the fact that it is the concentration of thyroxine itself which sets the general metabolic level. Thus, with increase in thyroxine concentration the rate constant for metabolism of many substances, including thyroxine as well as other hormones, is increased. Since the level of basal metabolism is controlled by the concentration of thyroid hormone in body fluids it would seem also that constancy of this function, which is more suitable to body economy than constancy of many other endocrine functions is best served by a relatively steady concentration and slow rate of degradation of thyroxine.

The association of persistently high levels of precipitable radioactivity with the presence of this radioactivity in the electrophoretically front running gamma globulin in the plasma of insulin treated subjects indicates an insulin-γ globulin complex, since the same electrophoretic patterns are demonstrable upon addition of insulin-I^{131} to the serum of these patients *in vitro*. Ultracentrifugal sedimentation of insulin-I^{131} with the serum globulins in these cases supports the conclusion that retention of insulin-I^{131} in the blood stream results from binding to the globulin and a consequent inability of the insulin molecule to pass rapidly through the capillary wall. Upon dissociation of the insulin-globulin complex, the relatively small insulin molecule either escapes into extravascular compartments or is again bound by a globulin molecule. The affinity of the globulin binding sites for the insulin is then the major factor in determining the rate of loss of insulin-I^{131} from the plasma in these subjects. In some cases this affinity was quite marked and the plasma concentration of insulin-I^{131} decreased slowly. In others the rate of fall of insulin-I^{131} in plasma was more rapid indicating an apparent lesser degree of affinity of the globulin for the insulin. However, the apparent affinity undoubtedly depends on the concentration of insulin present as is readily demonstrated by *in vitro* experiments with various insulin concentrations. Although insulin was withheld on the day of the studies from all diabetic patients receiving insulin, there may still have been some insulin remaining from the previous day's treatment. In some cases, then, differences in the quantity remaining may have played a role in the relative apparent binding affinities in different subjects. In the one insulin treated patient to whom two markedly different doses were given on successive days the curves of protein precipitable activity were superimposable (see legend to Figure 2) suggesting virtually identical binding and dissociation constants over a relatively wide range of insulin levels; however, if still higher levels of insulin had been achieved, a more rapid rate of disappearance from plasma might have been observed. It seems likely that the relative insensitivity of many insulin treated diabetic subjects to intravenously administered insulin during the glucose-insulin tolerance test (22) is attributable to binding by globulin.

The evidence presented in this paper indicates that an insulin binding globulin is acquired by human subjects in response to the administration of insulin. It would appear that the insulin binding globulin satisfies the requirements for an antibody as defined by Topley and Wilson (23): "An antibody is any substance which makes its appearance in the blood stream or body fluids of an animal, in response to the stimulus provided by the parenteral introduction of an antigen into the tissues, and reacts specifically with that antigen in some observable way." The reaction in this case is evidenced by the retardation of disappearance of insulin from the blood stream, by the altered migration in zone electrophoresis on paper and starch and by sedimentation with the globulins in the ultracentrifuge. This antibody does not appear to be a precipitating antibody even when concentrated five fold (no evidence of precipitation appears after 48 to 72 hours at 37° or up to 2 to 3 weeks at 4° C.) but merely binds the insulin.

It may be that the concentration of antigen-antibody complex is too low to be in the precipitable range or that the antibody is of the type generally regarded as "monovalent" or "incomplete" (24). Since complexing of insulin to the antibody of the present study does not lead to rapid removal from the circulation, but in fact serves to retain insulin within the circulation, the antibody has been here termed "insulin-transporting antibody." It should be noted that the relative electrophoretic mobility of the uncomplexed antibody is not known from these studies since an alteration in mobility may result following the complexing of insulin.

Whether the insulin-transporting antibody is of any clinical significance remains to be established. Its mere presence is certainly not necessarily related to the degree of insulin tolerance or intolerance presented by patients since all insulin treated patients apparently develop it in time. However, it is possible that fluctuations in its production may be causally related to the lability of insulin requirements in those patients who manifest what has been called "brittle diabetes." It is also quite possible, that in the presence of infections which call for heightened antibody response to bacterial or viral antigens, the increase in insulin requirements so frequently observed may likewise be related to altered production of insulin-transporting antibody. These possibilities represent fields for future investigation.

Whether this antibody in extremely high titer is the same antibody which has occasionally been identified in the rare instances of true insulin resistance is unknown. In the relatively small group which has been studied up to the present no definite relationship between insulin requirements and apparent magnitude of antibody binding of insulin has been established. However, a more quantitative expression of this antibody reaction is required than is available at present. The absence of insulin resistance in subjects who manifest marked binding of insulin-I^{131} to globulin is not unexpected. Since the total binding capacity is not unlimited, quantities of insulin in excess of that required to saturate the globulin binding sites might be expected to be available for metabolism at the same rate as insulin in subjects without antibody. The maximum amount of insulin bound to globulin observed in patients of this study was approximately 60 to 80 units as estimated from the specific activity of the administered insulin-I^{131} and the assumption that the volume of distribution of the globulin antibody is 2½ times the plasma volume as is the case with serum albumin (12).

Another possible consequence of insulin antibody production is to be considered, although it likewise remains purely speculative at present. This has to do with the possibility of a tissue reaction either to the circulating insulin-antibody complex or from tissue-fixed antibodies of similar nature. Possibly some of the complications of long standing diabetes which were unrecognized in the pre-insulin era, particularly the renal lesion responsible for the Kimmelstiehl-Wilson syndrome, may be causally related to long continued exposure to the circulating insulin-antibody complex.

These and many other problems, such as the longevity of insulin-antibody production after discontinuation of insulin therapy, which can most readily be studied in insulin treated schizophrenics, the degree of cross reaction with insulin of other species, particularly human insulin, and the kinetics of the insulin-antibody reaction, remain to be explored. The present study has not established the clinical significance of the insulin-transporting antibody and it may be that there is none to be established. The ubiquitous presence of such an antibody in insulin treated subjects, however, would appear to be a finding of interest.

SUMMARY AND CONCLUSIONS

1. Following intravenous administration of I^{131} labeled insulin to human subjects the time course of radioactivity in plasma has been studied by chemical and electrophoretic methods.

2. During the trace labeling of regular crystalline insulin with I^{131} some of the insulin is altered in an unknown fashion so that small but variable fractions of the radioactive material become bound to the serum proteins.

3. The radioactivity bound to serum proteins disappears from plasma much more slowly than unaltered insulin-I^{131}, so that within a short period of time following intravenous administration most of the protein precipitable radioactivity in plasma is not insulin-I^{131}.

4. During paper electrophoresis of plasma containing insulin-I^{131}, the labeled insulin is adsorbed

to the paper at the site of application, thus permitting separation from plasma protein bound radioactivity. The half time of disappearance from plasma of adsorbed radioactivity (insulin-I^{131}) after distribution in body fluids is of the order of 40 minutes which is in good agreement with the rate of degradation of insulin-I^{131} as estimated from the rate of appearance of non-precipitable radioactivity in the plasma.

5. In subjects treated with insulin for months to years the disappearance of insulin-I^{131} from plasma is much slower than in subjects never treated with insulin or treated with insulin for less than two to three months. It has been shown that this persistence of relatively high concentrations of insulin-I^{131} in the plasma of insulin-treated subjects is due to binding of insulin-I^{131} by an acquired globulin which satisfies the criteria for antibody. The insulin-"insulin transporting antibody" complex migrates in the front running γ globulin region on paper or starch block electrophoresis at pH 7.3 or 8.6. The binding of insulin to circulating globulin in these patients is confirmed by ultracentrifugation studies in which it is observed that insulin-I^{131} in the serum of insulin treated subjects sediments with the globulins but in the serum of control subjects sediments at a slower rate than serum albumin.

ACKNOWLEDGMENTS

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Addenda

(a) Since this paper was written, a report by Kallee and Seybold (Uber^{131} J-signiertes Insulin III, Zeitschrift Fur Naturforschung Bd. 1954, **9b**, 307) has come to our attention, in which shifting bands of radioactivity were observed in radioautographs of rat serum at various intervals following administration of I^{131} labeled insulin. Many of their unexplained observations appear to be resolved by the results of the present study.

(b) Since the preparation of this paper it has been established by two of us (R.S.Y. and S.A.B.) that the appearance of migrating components is a manifestation of radiation damage to the insulin. Similar changes have been produced by external irradiation with x-rays and radium at dose levels comparable to those delivered by the I^{131} present in these preparations.

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THE EFFECT OF SODIUM GLUTAMATE ON HEPATIC COMA [1, 2]

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Abnormalities of nitrogen metabolism may be important in the production of hepatic coma (1). This concept is supported by the following: 1) Elevated blood "ammonia" [3] concentrations have been described in patients with cirrhosis of the liver, many of whom were in hepatic coma; the highest values have occurred in comatose patients. 2) Some alcoholic patients with cirrhosis given nitrogenous substances including ammonium cation-exchange resins, ammonium salts, urea, and increments of dietary protein have developed impending hepatic coma (mental confusion and often the characteristic "flapping" tremor) which usually terminated after discontinuing these substances (2, 3). 3) A similar state occurred also in Eck fistula dogs and man after feeding increments of dietary protein and other nitrogenous substances (4–6).

Ammonium may react with glutamic acid to form glutamine or precursors of urea. Glutamic acid is used more than any other amino acid in brain energy metabolism, and has been reported to improve insulin coma in psychotic patients (7, 8). Walshe first used glutamic acid in the treatment of hepatic coma with encouraging results (9). Other investigators have since reported variable findings (10–20). This paper evaluates the effect of intravenous sodium glutamate administered to eleven patients with cirrhosis of the liver. Five were comatose, six showed signs of spontaneous [4] impending coma, and two had signs of impending coma which had been induced by ammonium salts.

MATERIALS AND METHODS

Patients. Five patients were men and six women, ranging in age from 34 to 67 years. In all but one, the diagnosis of cirrhosis was made by history of heavy alcohol and poor food intake coupled with physical and laboratory findings consistent with severe longstanding liver disease. The exception was a woman (A. R.) with presumed biliary cirrhosis. Nine patients died in hepatic coma and the diagnosis of cirrhosis was confirmed in seven at autopsy and in one by needle biopsy. In addition to cirrhosis one patient (J. H.) was found to have a co-existing chronic cholecystitis with cholelithiasis, another (J. W.) a small old encapsulated subdural hematoma, and a third (J. K.) bronchiogenic carcinoma with liver metastases as well as a primary carcinoma of the rectosigmoid and prostate. Four patients had hemolytic anemia thought to be consistent with their liver disease. (See Table I.) Guaiac positive stools were obtained in patient J. K. throughout treatment and patient A. P. vomited and aspirated guaiac positive material terminally, there was no evidence of gastrointestinal bleeding in the other patients.

Glutamate and ammonium salts. L-glutamic acid [5] was given as sodium glutamate already prepared by the manufacturer or prepared as follows: 20 grams of l-glutamic acid were neutralized with 20 per cent sodium hydroxide to a pH of 7.4 to 7.8 and made up to a volume of from 300 to 1000 ml. with sterile 5 per cent glucose in distilled water. This mixture, containing approximately 23 grams of sodium glutamate, was given intravenously during a 3 to 4-hour period in the afternoon to eight patients; three received from 103 to 355 grams continuously over a period of from 17 to 93 hours. The only reactions were anorexia and vomiting controlled by slowing the rate of infusion. Di-ammonium citrate was given in 4-gram doses five times during the day (daily dosage of 20 grams). Ammonium chloride was given in half-gram enteric-coated tablets, 2 grams every two hours during the daytime, to total 10 grams daily.

[1] This work was supported in part by the Office of The Surgeon General, Department of the Army, and in part by a grant from Merck and Company, Inc., Rahway, New Jersey, to Harvard University.

[2] A preliminary report of part of this investigation is included in the Proceedings of the Forty-Sixth Annual Meeting of the American Society of Clinical Investigation, Atlantic City, May 4, 1954 (J. Clin. Invest., 1954, 33, 971).

[3] Whether free ammonium exists in shed blood or forms after shedding is argued. If it does exist it is present as ammonium ion. Therefore, when referring to plasma or blood, we have put the word ammonia in quotes.

[4] "Spontaneous" is the term used to describe coma or impending coma in which precipitating factors, except severe liver disease, were not known.

[5] Kindly provided by Dr. Augustus Gibson, Merck & Co., Rahway, New Jersey.

Diet. Patients were maintained on 2000 to 2400 calorie diets with not more than 66 grams of protein. Only patient J. H., who had undergone a previous period of protein depletion without marked beneficial response, received less protein during treatment than before. Because nine patients had ascites and ten had edema, daily dietary sodium intake was restricted to approximately 200 milligrams. Patients unable to eat received a similar diet by tube or 10 to 15 per cent glucose by vein. Twenty-four-hour fluid intake was adjusted to equal the estimated fluid lost with occasional exceptions when a water load was given.

Laboratory methods. The total 24-hour urine volumes were recorded. Blood was drawn about 1 to 2 hours after breakfast. Serum determinations were carried out as follows: Carbon dioxide content as described by Van Slyke and Neill (21); sodium and potassium concentrations by internal standard flame photometry (22); chloride concentration by the iodometric titration of Van Slyke and Hiller (23); and urea nitrogen concentration according to Owings and Mandel (24). Liver function studies including 45-minute serum bromsulphalein retention, cephalin flocculation, thymol turbidity and flocculation, and serum bilirubin concentrations were performed at weekly intervals using standard methods. Icterus index, hematocrit, and urine urobilinogen were measured at more frequent intervals.

The blood "ammonia" content, expressed as ammonia nitrogen (NH_3–N), was determined by a modification of the Seligson method (25) in the first eight patients and by McDermott and Adams' (6) modification of the Conway technique in the last three. Further modifications of the Seligson technique were: 1) The determination was always done on freshly collected plasma and 2) rotation was carried out for a period of one hour at 28° C. instead of 23° as described. Values for volatile plasma base by this method were lower than those obtained by Kirk (26) but in the same range as those obtained by Seegmiller, Schwartz, and Davidson (25). Daily variations were marked but duplicate samples checked well. The second technique utilized whole blood instead of plasma, and a ten-minute time limit was employed for diffusion of volatile blood base into the acid center well. Here, values ranged from 0.25 to 0.7 gamma of NH_3–N per milliliter of normal blood and from 0.35 to 1.0 gamma per milliliter of blood in patients with cirrhosis without mental symptoms. Of the two ammonia methods used, the second (McDermott and Adams' modification (6) of the Conway technique) has yielded lower values and has proved much more reproducible and reliable in our hands although its results do not consistently reflect changes in consciousness. NH_3–N determinations were done daily in the mornings on the first eight patients who received no more than 23 grams of sodium glutamate per day; determinations were done much more frequently in the last three cases who received larger amounts.

Clinical evaluation. The state of consciousness, the

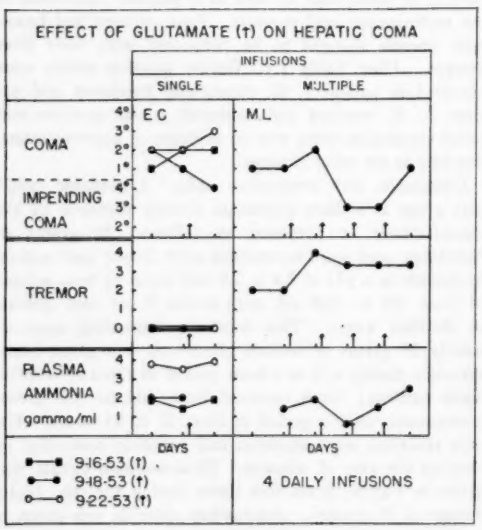

FIGURE 1

Two patients were given 23 gm. of sodium glutamate as indicated by the vertical arrows, E. C. one infusion on three separate occasions on the dates shown (left of figure), and M. L. four consecutive daily infusions (right of figure). Plasma "ammonia" is expressed as NH_3-nitrogen.

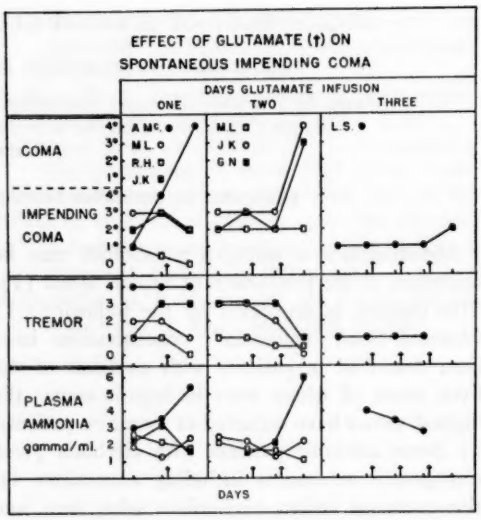

FIGURE 2

Twenty-three gm. of sodium glutamate, as indicated by the vertical arrows, were given to 4 patients on a single occasion (left of figure), to 3 patients on two consecutive days (center of figure), and to 1 patient on three consecutive days (right of figure). Plasma "ammonia" is expressed as NH_3-nitrogen.

most reliable method for evaluating hepatic coma, was classified daily into sub-groupings of coma or impending coma to document accurately change and to allow comparison from patient to patient. Many well-known signs, *i.e.*, fetor hepaticus, abnormal plantar and deep tendon reflexes, ankle clonus, flaccidity, convulsions, etc. were not included because none of these occurred consistently at a given depth of coma. This classification is not complete; also some cases of hepatic coma will not fit precisely into the pattern described (*27*).

Two main divisions were set-up—impending hepatic coma (including both spontaneous impending coma and impending coma induced by ammonium salts), and hepatic coma. The former was characterized by confusion and disorientation and the latter by loss of consciousness.

Impending coma was divided into five subheadings as follows:

1. *Questionable impending coma.* The patient often "did not feel quite right," exhibited a change in emotional tone, or did not possess his usual mental agility. Obvious confusion was present only at night.

2. *First degree impending coma.* Reasoning, such as ability to perform calculations, was impaired but orientation as to time was present. Often a change in personal tidiness and a "far away look" (28) in the eyes were observed.

3. *Second degree impending coma.* Response was delayed and confusion was easily brought out by simple conversation. Orientation was present to place but not to time.

4. *Third degree impending coma.* Response to questioning was grossly inaccurate, delayed, and slow; simple phrases were used. Somnolence was apparent; orientation to place and time was absent.

5. *Fourth degree impending coma.* Drowsiness and confusion were marked; spoken response was delayed, slow, irrelevant, monosyllabic, and often incoherent.

Coma was divided into four subheadings: Spoken response was absent, unlike impending coma.

1. *First degree coma.* Responses to spoken voice, such as motion, grimacing, groaning, or turning the eyes, were observed.

2. *Second degree coma.* Response was absent to voice but present to painful stimuli which produced motion, change in respiration, and perhaps grimacing. Corneal and gag reflexes were present.

3. *Third degree coma.* Painful stimuli produced, if any response, only a change in respiration. Either or both corneal and gag reflexes were present.

4. *Fourth degree coma.* Painful stimuli produced no response; gag and corneal reflexes were absent.

A common sign particularly in impending hepatic coma is the "flapping tremor" described by Adams and Foley in 1949 (*29*). This is a coarse asynchronous flexion-extension movement occurring in one or more muscle groups depending on its severity. It is best demon-

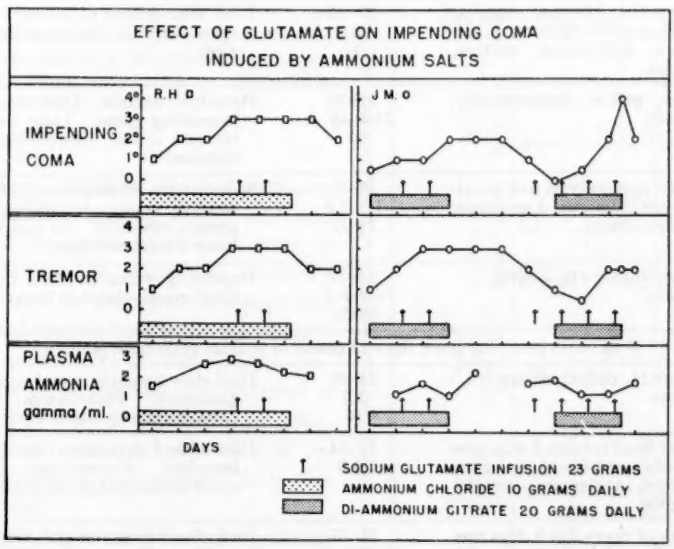

FIGURE 3

Twenty-three gm. of sodium glutamate, indicated by the vertical arrows, were given on two consecutive days to patient R. H. along with ammonium chloride (left of figure), and to patient J. M. both before and during di-ammonium citrate administration (right of figure). Plasma "ammonia" is expressed as NH_3-nitrogen.

strated by spreading the fingers and hyperextending the hand at the wrist with the pronated arm resting on the bed. In comatose patients the tremor often is elicited only when the hand is passively flexed at the wrist or held by the observer; it is not elicited when the limb is flaccid. The tremor was graded from 1+ to 4+ as follows:

1+ Definite tremor was present only with outstretched arms resting on the bed and hands hyperextended as described.

TABLE I

Clinical and laboratory data on the 11 patients with cirrhosis of the liver

Patient Age Sex	History and physical signs	Laboratory (range of values) Hematocrit % Serum bilirubin (*mg./100 ml.*) Bromsulphalein (% retention)‡	Disposition and comments
8 patients receiving a maximum of 23 grams of sodium glutamate per day			
M. L. 64 M	*Icterus, angiomata, pallor, hepatomegaly, ascites, edema.	24–29 5.3–10.0 35–57	Hemolytic anemia. Died after 33 days of coma.† Postmortem: Laennec's cirrhosis, arteriosclerotic heart disease.
E. C. 51 F	Paracentesis 9–8–53. Marked ascites and edema.	29–35 1.7 53	Died 9–24–53 after 14 days of coma.† Postmortem: Laennec's cirrhosis.
J. K. 67 M	History of NH_4Cl ingestion and diarrhea. Icterus, angiomata, hepatomegaly, ascites, edema.	47–51 5.7–9.0 46	Stools benzidine positive. Abnormal chest and barium enema films. Died after 16 days coma.† Postmortem: Laennec's cirrhosis. Primary carcinoma of the bronchus, rectosigmoid, and prostate.
G. N. 49 M	*Icterus, angiomata, hepatomegaly, ascites, edema.	46–50 19.2	Died after 7 days of coma.† No postmortem or liver biopsy obtained.
A. Mc. 54 F	History G.I. bleeding stopping 7 days prior to coma.† Icterus, angiomata, ascites, edema.	29–32 22.8 57 —	Died after 4 days of coma.† Postmortem: Laennec's cirrhosis, hypertensive cardiovascular disease.
L. S. 37 F	*Icterus, pallor, hepatomegaly, edema.	25–26 24.4–46 67	Hemolytic anemia. Died suddenly after 6 days of impending coma. Liver needle biopsy: Great increase in fat, little fibrosis. No postmortem obtained.
R. H. 49 M	History paracentesis and paraldehyde. Icterus, angiomata, ascites, edema.	38–49 1.3–2.0 29–51	Spontaneous improvement after 20 days of impending coma. Impending coma induced by ammonium salts. No symptoms of coma since. Liver biopsy not done.
J. M. 34 M	*Icterus, angiomata, ascites, edema.	33–39 6.1–8.2 38–53	Impending coma induced by ammonium salts. Died months later in hepatic coma.
3 patients receiving more than 23 grams of sodium glutamate per day			
J. H. 49 F	*Angiomata, pallor, ascites, edema.	24–26 2.9 36	Died after 4 months coma† and 5 days after sodium glutamate. Postmortem: Laennec's cirrhosis, chronic cholecystitis and cholelithiasis.
J. W. 46 F	*History head trauma 6 wks. previously and 1 day of confusion. Icterus, angiomata, hepatomegaly.	32–34 24.5 —	Died after 5 days coma† during glutamate administration. Postmortem: Laennec's cirrhosis, small encapsulated subdural hematoma.
A. P. 67 F	History of operation 9 days previously and leakage of ascitic fluid. Icterus, angiomata, hepatomegaly, ascites.	34–37 24 —	Died after 7 days coma† during glutamate administration. Postmortem: Biliary cirrhosis, blood in upper gastrointestinal tract, aspiration of stomach contents.

* Only factor known to precipitate coma was severe liver disease.

† Onset of coma dated from the onset of confusion.

‡ Per cent retention of B.S.P. measured in the serum 45 minutes after 5 mg. of dye per kilogram body weight was injected intravenously.

2+ The tremor was present with arms held outstretched off the bed and hands dorsiflexed at wrists. A similar tremor was elicited when the feet were dorsiflexed.

3+ Tremor was prominent in the hands, feet, and also was present in other muscle groups.

4+ The tremor was sufficiently severe to interfere markedly with voluntary motor acts.

According to their states of consciousness, the first eight patients who received a maximum of 23 grams of sodium glutamate per day were placed in one of three groups on the day of the initial glutamate infusion as follows: 1) coma, 2) spontaneous impending coma, 3) induced impending coma (see Figures 1–3). Those who exhibited first to fourth degree coma made up Group 1. Those who exhibited impending coma without precipitating factors made up Group 2. The two patients who received ammonium salts with subsequent development of tremor and confusion comprised Group 3. All patients remained in their original group until observations following single or multiple infusions of sodium glutamate were completed. If a lag of one or more days took place between glutamate infusions, the patient was regrouped if warranted (see patient M. L.).

None of these eight patients received adrenal steroid hormones, supplementary potassium salts, or antibiotics. The three remaining patients who received large quantities of glutamate were all comatose and were classified into subheadings of coma as previously described. Two of these, E. C. and A. P., received supplementary oral potassium salts and one, A. P., received cortisone and antibiotics in addition to glutamate. With two exceptions, R. H. and E. C., infusions were administered when confusion or coma was either progressive or stabilized in all eleven patients.

RESULTS

Pertinent history, physical signs, hematocrit, serum bromsulphalein retention, serum bilirubin, and disposition of the eleven patients are given in Table I. Table II presents clinical and laboratory data before and after the glutamate infusions and Figures 1, 2, and 3 present by groups the changes in mental state, tremor, and plasma NH_3–N concentrations of the eight patients who received a maximum of 23 grams of glutamate per day. The remaining three comatose patients who received larger quantities of glutamate are later presented in more detail.

Two of the first eight patients, one receiving glutamate on three separate occasions and the other on four consecutive days, were comatose. Neither patient demonstrated any consistent changes in mental status, tremor, or plasma NH_3–N levels. In the impending coma group,

TABLE II—*Effect of 23 gm. daily quantities of intravenous sodium glutamate on the clinical and laboratory data of 8 patients with cirrhosis of the liver*

	Patient	Dates of glutamate infusions (inclusive)	Dates of NH_4 salt administration (inclusive)	Mental status*		Tremor		Plasma ammonia-N *gamma/ml.*		Serum sodium *mEq./liter*		Serum potassium *mEq./liter*		Serum chloride *mEq./liter*		Serum carbon dioxide content *mEq./liter*		Serum urea nitrogen *mg. %*		Urine volume *ml. in 24 hrs.*	
				Before†	After‡	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
Hepatic coma	E. C.	9/16/53		2° C	2° C	0	0	1.4	1.9	135	136		4.6	93	97	28	26	42	44	500	600
		9/18/53	None	1° C	4° I.C.	0	0	1.9		133	129	4.7	5.5	101	99	27	30	50	59	600	320
		9/22/53		2° C	3° C	0	0	3.6	4.0	132	131		5.0	94	98	26	27	59	88	500	
	M. L.	10/19–10/22/53	None	1° C	1° C	2+	3+	1.4	2.5	137	138	3.4	3.9	92	94	33	39	18	34	500	630
	M. L.	9/30/53	None	3° I.C.	2° I.C.	2+	1+	1.3	2.4	132	136	4.0	3.1	93	93	28	31	20	25	1,380	500
		10/6–10/7/53		2° I.C.	2° I.C.	1+	±	1.7	1.1	122	134	2.7	3.4	92	94	32	33	23	23	860	760
Impending hepatic coma	J. K.	9/9/53	None	3° I.C.	2° I.C.	3+	3+	3.1	1.7		125		3.6				28		18		
		9/15–9/16/53		3° I.C.	4° I.C.	3+	0	2.3	2.3	137	132	3.6	4.1	88	88	34	34	20	55		
	G. N.	9/14–9/15/53	None	3° I.C.	3° C	3+	1+	1.6	6.3	129	122	3.5	3.4	91	95	26	30	40	66	290	70
	A. Mc.	10/27/53	None	3° I.C.	4° C	3+	0	3.6	5.4	130	131	4.0	3.1	86	94	29	29	11	17		
	L. S.	11/5–11/7/53	None	1° I.C.	2° I.C.	1+	1+	4.0	2.8	124	129	4.1	3.0	84	83	17	26	8	25	300	40
	R. H.	8/12/53	None	1° I.C.	0° I.C.	1+	0	2.2	1.8	140	137	4.9	5.0	92	91	24	27	57	58	940	920
Induced impending hepatic coma	R. H.§	8/25–8/26/53	8/19–8/26/53	3° I.C.	3° I.C.	3+	3+	2.9	2.3	126	125		4.9	115	112	13	15	27	29	470	200
	J. M.‖	10/14–10/15/53	10/13–10/15/53	1° I.C.	2° I.C.	2+	3+	1.2	1.1	136	141	3.9	4.0	104	105	31	31	10	10	2,100	300
		10/19–10/22/53	10/20–10/22/53	1° I.C.	4° I.C.	2+	2+	1.7	1.8	146	142	4.2	4.9	107	105	30	28	9	10	670+	620+

* C = Coma, I.C. = Impending Coma.
† Before—Morning of the glutamate infusion day.
‡ After—Morning after glutamate infusion.
§ Impending coma induced with ammonium chloride 10 gm. per day.
‖ Impending coma induced with di-ammonium citrate 20 gm. per day.

four patients received a single glutamate infusion, three patients an infusion each of two successive days, and one patient three infusions at daily intervals. After glutamate three of these patients became worse and died, three improved slightly, and one showed no change in mental status. Tremor remained the same or decreased in all of this group and plasma NH_3–N concentrations rose in two, remaining essentially unchanged in the others.

Two patients in whom impending coma was induced by oral ammonium salts were given infusions of sodium glutamate in addition to their ammonium salts. Patient R. H. exhibited no change in confusion or tremor although the plasma NH_3–N levels fell slightly after glutamate. Patient J. M. became more confused and his tremor also became accentuated despite two consecutive daily infusions of glutamate. Later, glutamate, given prior to and during administration of di-ammonium citrate, failed to prevent the onset and exacerbation of impending coma or accentuation of tremor in the same patient.

The results of the other laboratory determinations performed on the first eight patients are given in Tables I and II. Serum sodium and potassium concentrations were often lower than normal but consistent changes did not occur after sodium glutamate. Likewise, no consistent variations occurred in serum chloride concentrations which were initially low in most patients and high only in the patient receiving ammonium chloride. Serum carbon dioxide contents rose more than 3 mM per L. only in three patients who received sodium glutamate for at least two days. Serum urea concentrations rose more than 15 mg. per cent in five patients, two of whom had become markedly oliguric.

The first patient, J. H., received 103 grams of sodium glutamate in 17 hours, 75 grams of which were given during the first 10 hours. Her blood NH_3–N, initially markedly elevated (3.45 gamma per ml. of blood), dropped promptly to within normal limits where it remained on 12 occasions until 4½ hours after glutamate was discontinued, when it rose again to abnormal levels. Her mental status, initially second degree coma, improved to first degree coma after two hours, and to fourth degree impending coma just as the infusion was discontinued. She then improved further to third degree impending coma where she remained for 3 days until two days before death. The 2 + tremor disappeared during and after glutamate therapy. Biochemical changes observed during her treatment were a drop in serum potassium concentration from 4.3 to 3.2 mEq. per l., a rise in serum carbon dioxide content from 25 to 38 mEq. per l., and a rise in serum sodium concentration from 134 to 147 mEq. per l. Twenty-four-hour urine sodium output remained the same as previously (approximately .5 to 1.5 mEq.), potassium rising from 20 to 60 mEq. per 24 hours on the day of sodium glutamate administration.

The second comatose patient, J. W., received 175 grams of sodium glutamate over a period of 35 hours, and improved from third degree to second degree coma in 12 hours. During this period she developed twitching of the arms and legs. Following this short period of improvement she developed oliguria, hypotension, slipped into fourth degree coma, and died. Blood NH_3–N concentrations, determined on six occasions, were normal before and remained normal during glutamate therapy. The tremor was not present during her comatose state. Despite administration of 180 mEq. of intravenous potassium, her serum potassium concentration dropped from 3.2 to 1.5 mEq. per l. during sodium glutamate treatment. Serum sodium concentration rose from 125 to 132 mEq. per l. and carbon dioxide content was found to be 36 mEq. per l. after glutamate. Urine collected during the last 14 hours of life contained 190 mEq. of sodium and 77 mEq. of potassium.

At the start of glutamate therapy, the third patient (A. P.) had ascitic fluid drainage from an open abdominal wound created during an operation 9 days previously. This patient received hydrocortisone and antibiotic medication in addition to sodium glutamate; hydrocortisone was discontinued 3 days prior to death. A total of 355 grams of sodium glutamate were given over a 93-hour period and the blood NH_3–N concentration dropped from 2.6 gamma per ml. and remained within normal limits, except for two slightly elevated levels both of 1.15 gamma per ml. obtained 22 and 19 hours prior to her death. The last determination was performed 15 hours before death. This patient exhibited improvement in mental state from third degree coma to first degree coma within 20 hours of starting glutamate,

hydrocortisone, and antibiotics. Later the mental state fluctuated between third and fourth degree impending coma until 20 hours before death when hypotension, previously relieved by position, became pronounced. She then became oliguric and coma progressed to death. Terminally, guaiac positive material, some of which she aspirated, was obtained from the stomach. Tremor, absent in the initial comatose state, reappeared during improvement and finally disappeared during the last few hours of life. Again there was a drop in serum potassium (from 3.6 to 2.7 mEq. per L.) despite administration of 220 mEq. of potassium during her treatment. She lost 67 mEq. of potassium in the urine over two of the four days she was treated. Urine sodium loss was less than 2 mEq. per day but a large amount was lost in the ascitic fluid (estimated loss 1 to 4 liters daily). Potassium loss in the ascitic fluid would have been comparatively slight.

DISCUSSION

Examination of mental state, the best method for describing degree or depth of coma, was used to evaluate the therapeutic efficacy of the sodium glutamate. The method is subjective, however, and daily or even hourly variations in the state of consciousness often occur. The prognosis of coma precipitated by known factors, such as ammonium-containing compounds, other drugs, high nitrogen intake, gastro-intestinal hemorrhage, infection, paracentesis, delirium tremens, and certain electrolyte disturbances, is almost certainly less severe than that of spontaneous hepatic coma (30). Also, when any of these precipitating factors is present, it is very difficult to evaluate a substance of unknown therapeutic value. Steps taken to insure an accurate evaluation of sodium glutamate's efficacy in hepatic coma included the following. Patients were seen a minimum of two to three times daily by the same observer and were checked by others. Glutamate was administered (with two exceptions already mentioned) during a period when the depth of coma was increasing or when the state of consciousness appeared stabilized. If any factors known to precipitate hepatic coma were present, the patient was observed for at least one week until the mental state probably did not reflect the precipitating factors and only then was glutamate given (exception was patient A. P.). Patients were observed before the development of impending coma with three exceptions, and these were seen initially in impending coma. No other medications except supplementary potassium, cortisone, and antibiotics were given and these only to the patients mentioned. Therefore, with the exception of patient A. P., it seemed likely that any improvement resulted from sodium glutamate although the possibility of a spontaneous change cannot be eliminated entirely.

The presence of the tremor was useful in suggesting that confusion would soon appear. Fluctuations in confusion, when present, were not always paralleled by the severity of the tremor. The latter, often but not always absent in coma, may appear with improvement to impending coma (patient A. P.).

Our results indicate that 23 grams of sodium glutamate intravenously had little effect on the mental state, tremor, or plasma NH_3–N concentrations of the first eight cirrhotic patients studied. Because, contrary to our findings in these eight patients, glutamate has been reported to lower venous blood "ammonia" levels and improve coma in some patients with chronic liver disease, larger quantities of this compound were tried in three patients with temporary improvement in mental state. No conclusions could be drawn in the third patient (A. P.), who also received adrenal steroids and antibiotics, except that glutamate did not prevent an eventual fatal outcome. A similar fate befell the other two patients; one expired while still receiving glutamate and the other five days after its discontinuance. The blood NH_3–N concentrations were kept at or close to normal in all three patients during glutamate therapy, falling promptly from an initial elevation in two and rising 4½ hours after its discontinuation in one. This rise shortly after discontinuance of glutamate may explain the lack of significant change in plasma NH_3–N concentrations in the first eight patients because determinations were done 14 to 18 hours after glutamate was discontinued.

Electrolyte disturbances might limit or otherwise effect the beneficial effect of a trial medication, although abnormalities were not stiking in the first eight patients who received only 23 grams of sodium glutamate daily. In the remaining three patients the main complication of administering large

quantities of sodium glutamate was the development of hypokalemic alkalosis. Although the pH of the serum was not measured, there were elevations in serum carbon dioxide contents and sodium concentrations consistent with this abnormality in all three instances. The first patient experienced a marked drop in serum potassium, an increase in urinary potassium excretion, and did not receive potassium salts. Serum potassium concentrations also fell in the other two, despite terminal oliguria and administration of supplementary oral potassium in excess of their urinary potassium excretion. This latter finding seems not completely explained by the administration of large quantities of sodium although it has been shown in dogs that administration of sodium chloride may cause increased urinary potassium excretion (31). Administered glucose in large amounts with insulin present will often decrease serum potassium concentrations and might explain the low concentrations seen here. Despite the fact that these patients all received glucose this latter explanation alone seems doubtful because the severe hypokalemias did not respond to potassium administration, which is unusual in our experience. Although balance data are lacking, an additional possible explanation is that intracellular potassium may have increased during glutamate therapy accounting in part for the low serum potassium concentrations. We have not yet evaluated the effect of glutamic acid given as the potassium or magnesium salt.

The way in which large quantities of glutamate produced temporary improvement in hepatic coma remains obscure. Three possibilities exist as discussed by Weil-Malherbe (8). Because glutamate is necessary for maintenance of potassium gradients in guinea pig brain slices (32) one might wonder whether this action as well as the shown blood "ammonia" lowering effect produced mental improvement. A third effect of glutamic acid is to produce an adrenergic response (8). Whether this effect would account for improvement in hepatic coma is not known although some believe it is responsible for the favorable action of glutamate in insulin coma.

In summary it is realized that although results obtained with sodium glutamate were discouraging here and have been found so by others, the criteria set up for its evaluation were rigid. Its use may be more encouraging in other situations, *i.e.*, hepatic coma precipitated by NH_4 salts, a high protein intake, gastrointestinal hemorrhage, and possibly other factors. Good results have been observed in so-called "meat intoxication" of patients with portacaval shunts (33) and might also be found in patients with chronic liver disease and extensive collateral circulation in whom a somewhat similar situation exists.

CONCLUSIONS

1. Sodium glutamate, 23 grams intravenously daily, given to 8 of 11 patients with cirrhosis, failed either to affect favorably the course of spontaneous impending hepatic coma and coma or to prevent the onset or exacerbation of impending coma induced by ammonium salts. Plasma NH_3–N concentrations showed no consistent change on the day following glutamate.

2. Much larger quantities of intravenous sodium glutamate had a temporary beneficial effect on the coma of two and possibly three other patients with cirrhosis. Peripheral venous blood NH_3–N concentrations remained or became normal during the therapy.

3. A clinical method for evaluating the depth of hepatic coma or impending coma was found necessary and hence was developed and evaluated.

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IMMUNITY IN VOLUNTEERS RECOVERED FROM NON-BACTERIAL GASTROENTERITIS [1]

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The afebrile type (1) of acute infectious nonbacterial gastroenteritis seems to be an extremely common diarrhea in the Northeastern United States. It occurs yearly in some families and communities (2, 3). Whether the recurrences are due to the early waning of immunity or to the existence of immunologically distinct agents has not been determined. Following an attack of gastroenteritis evoked by the Marcy strain (4), which is presumably a virus, an effective immunity develops in volunteers (1, 4–6), and in an earlier report the opinion was expressed that immunity probably does not persist for long (5). The present study shows that individuals who recovered from experimental Marcy gastroenteritis more than a year before were fully resistant to reinfection. It may well be, therefore, that the second explanation of the recurrences is the correct one and more than one antigenically distinct agent exists.

METHODS

Afebrile nonbacterial gastroenteritis can be highly contagious; numerous large epidemics have been reported (4, 5, 7, 8). Like the experiments previously done with the Marcy strain, therefore, this study was carried out in a closed state correctional institution, where volunteer subjects were individually isolated in well-equipped cells for periods of several weeks. Stringent precautions against accidental spread of the disease were enforced under nursing supervision.

Volunteers were healthy male inmates 21 to 30 years old who had no known exposure to gastroenteritis prior to their entry into the study. Their psychological stability was assured; almost all had undergone previous inoculation with control specimens such as autoclaved fecal suspensions or broth (6) or had participated in other oral inoculation experiments unrelated to nonbacterial gastroenteritis (9). The symptoms and signs they exhibited were therefore unlikely to be due to suggestion. Their feces contained no known enteric pathogens.

The inoculum was a supernate of watery feces collected from Volunteer 61 (T. H.), one of a group representing the 6th serial passage of the Marcy strain in volunteers. At 12:30 p.m. on July 2, 1950, he had abrupt onset of nausea and vomiting, followed by malaise, vertigo, and 27 episodes of watery diarrhea within the next 36 hours. Fecal specimens from eight bouts of diarrhea occurring between 7 a.m. and 6 p.m. on July 3, 1950, were collected in sterile glass screw-capped jars and frozen for storage at −70°C. within 10 minutes after the patient produced them. On April 12, 1951, they were thawed at ∽37°C. and centrifuged horizontally at 3000 r.p.m. at ∽4°C. for 45 minutes. The sediment was discarded and the supernate recentrifuged similarly for 30 minutes. The second supernate was thoroughly mixed, dispensed in 10-ml. amounts to 40 jars, and refrozen in the CO_2 box. Microscopic and cultural examination revealed no bacteria, and no infectious agent was detected by inoculation of tissue cultures and experimental animals, including monkeys and suckling mice.

The content of an appropriate number of jars was thawed from time to time for the experiments reported in the text. Some previous results with this inoculum have been published (1, 6). The inoculum for the present study was composed of different specimens than the pool made up of stools collected at other times from the same patient, employed for another investigation (10).

As in preceding studies (1, 4), inoculum was fed mixed with milk, and fecal specimens from each ill volunteer were inspected. Some specimens were collected for subsequent bacteriologic examination and inoculation of animals or tissue cultures.

Criteria for recognition of experimental illness were more strict than those employed in an early experiment (5). Experience has shown that the most common manifestations of the induced disease are watery diarrhea, abdominal cramps and pain, anorexia, nausea, and vomiting (1, 4, 6). In this study the diagnosis of Marcy gastroenteritis was based on the presence of watery diarrhea plus at least two of the other symptoms or signs. Anorexia could be objectively noted by physicians,

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TABLE I

Results of initial inoculations with stored aliquots of the inoculum from Volunteer 61

Inoc. No.*	Date inoc.	Duration of storage since collection on July 3, 1950		Dose *ml.*	No. of volunteers	Cases of gastro-enteritis
		Years	*Days*			
1(1)	12.17.51	1	168	7	7	6
3(1)	1.15.52	1	197	7	8	7
4(1)	6.23.52	1	357	7	1	1
5	3.28.53	2	270	1	8	3
7	4.17.53	2	290	7	6	6
8	5.18.53	2	321	3.5†	6	4
9	5.29.53	2	333	3.5‡	6	4
Totals: Volunteers fed 1 ml.					8	3
Volunteers fed 3.5 ml.					12	8
Volunteers fed 7 ml.					22	20
All volunteers					42	31

* Refers to occasions on which aliquots were selected at random, mixed, pooled, and used. (Inoculation 2 was an immunity test done approximately two weeks after onset of gastroenteritis and is omitted.)

† Mixed with 3.5 ml. of acute-phase serum pool. See text.

‡ Mixed with 3.5 ml. of convalescent-phase serum pool. See text.

nurses, or attendants since the volunteers were tray-fed; it could therefore be judged either as a sign or a symptom. The lack of symptoms and signs exhibited by these and other volunteers given noninfectious or sham inoculations under the conditions of the experiments has already been recorded (1, 6).

RESULTS

Various experiments in which volunteers from New York State correctional institutions were fed aliquots of the pooled inoculum from Volunteer 61 (see Methods) had shown that in spite of long storage in the dry ice box the inoculum continued to be highly infectious for previously uninoculated men (Table I, Inoculations 1, 3, 4, 5). Four subjects who had contracted the disease approximately 15 months before and one who had suffered an induced illness 9 months previously, revolunteered to be reinoculated to test whether they were immune (Table II). All five had exhibited the diarrheal syndrome of afebrile nonbacterial gastroenteritis with characteristic incubation periods after ingestion of initial doses of 7 ml. of the bacteria-free fecal supernate from Volunteer 61, portions of which were still on hand. Their illnesses have been mentioned in a published report and the least severe of them, Case E. H. (Volunteer 89), is among those represented graphically (1). The five men had remained well in the interim between their first exposure and their recruitment for reinoculation.

The design of the immunity trial was dictated by opportunity. The number of volunteers avail-

TABLE II

Reinoculation of five recovered volunteers with aliquots of the inoculum from Volunteer 61 to test active immunity

Initial inoculation (7 ml.) and illness				Reinoculation (7 ml.) to test active immunity				
						Interval since onset		
Volunteer	Inoc. No.*	Date inoc.	Onset of gastro-enteritis	Inoc. No.	Date inoc.	*Years*	*Days*	Result
86 (H. W.)†	1	12.17.51	12.20.51	6	4.10.53	1	112	No illness‡
73 (A. B.)	3	1.15.52	1.17.52	6	4.10.53	1	84	No illness
74 (A. E.)	3	1.15.52	1.16.52	6	4.10.53	1	85	No illness
78 (G. S.)	3	1.15.52	1.17.52	6	4.10.53	1	84	No illness
89 (E. H.)	4	6.23.52	6.27.52	6	4.10.53	0	287	No illness

* See Table I for explanation.

† This volunteer experienced a reinoculation with a 7-ml. dose of an aliquot of the same inoculum, on 1.2.52, and remained well (1).

‡ No symptoms or signs.

TABLE III

Tests of significance of differences between results of immunity trial and those of initial inoculations

	I	II	III	IV	V
Inoculation No.*	6†	7	8 and 9	7, 8, and 9	1, 3, and 4
Type	Reinoculation	Initial inoc.	Initial inoc.	Initial inoc.	Initial inoc.†
Dates	4.10.53	4.17.53	5.18.53 5.29.53	4.17.53 5.18.53 5.29.53	See Table I
Dose	7 ml.	7 ml.	3.5 ml.	7 ml. or 3.5 ml.	7 ml.
Not ill	5	0	4	4	2
Ill	0	6	8	14	14
P		0.0022	0.0204	0.0037	0.0010

* See Table I for explanation.

† The five volunteers reinoculated on the occasion of Inoculation No. 6 were among the 14 subjects who became ill following initial inoculations 1, 3, or 4 (Column V).

able to serve as controls was uncertain. We proceeded with the reinoculation on the premise that if a sufficient number of reinoculated men became ill, demonstration of the infectivity of the inoculum would be unnecessary, while if they did not become ill, the inoculum could subsequently be tested for infectivity in additional volunteers exposed for the first time. This was thought permissible because the inoculum to be used throughout was pooled and mixed before it was stored in 10-ml. aliquots, and the vials employed for each inoculation were selected at random from the dry ice box when needed.

The reinoculation consisted of feeding 7 ml. of pooled aliquots of the inoculum to each of the five men. This was the same dose of the same inoculum they had received before. None showed any symptoms or signs of illness (Table II).

During the next 2 months remaining aliquots of the inoculum were tested for potency in 3 new groups of subjects, none of whom had had prior exposure to Marcy gastroenteritis inoculum or known contact with the natural disease (Table I, Inoculations 7–9). Inoculation 7 was done in the usual way, but for Inoculations 8 and 9, serum was mixed with the fecal supernate. The reasons for this will be discussed later.

Each of the six volunteers fed a 7-ml. dose of fecal inoculum alone developed typical gastroenteritis (Table I, Inoculation 7). Of six men fed 3.5 ml. mixed with an equal volume of acute-phase serum from Marcy gastroenteritis volunteers, four became ill (Inoculation 8). The same outcome followed ingestion of 3.5 ml. of inoculum mixed with serum from volunteers convalescent from Marcy gastroenteritis (Inoculation 9). Each of the 14 cases that occurred among these 18 men fully met the criteria for diagnosis outlined above. A few were severe, most of them were of moderate severity, and a few were mild. An example of the latter is the case of W. B. (Volunteer 127), who swallowed 7 ml. of inoculum, developed mild abdominal cramps and borborygmi with watery diarrhea after an incubation period estimated at 64 hours, experienced anorexia and headache on the following day, during which the feces continued to resemble pea soup, then recovered.

The challenge of the five recovered volunteers was done 728 days and the three trials of infectivity were carried out 735, 766, and 777 days, after the fecal supernate had been pooled, mixed, and split into 10-ml. portions. The dose for Inoculations 8 and 9 was half that employed in the challenge of the recovered volunteers. Under these circumstances the attack rates obtained in the potency tests might tend to be lower than those expected if the infectivity of the inoculum had been determined in control subjects given the same dose at the same time as that fed the five men in the immunity trial. In spite of this possible bias, tests of significance show that the differences between the results of the immunity and potency trials respectively are highly unlikely to have occurred by chance variation alone (Table III).

If a 2 by 2 table is formed with the outcome of the immunity trial (Table III, Column I) and that of the potency test of an equal dose of 7 ml. (Column II) the value of P as used in the "exact treatment" (11) is 0.0022.[4] If the pooled results of initial inoculations with the 3.5-ml. dose (Column

[4] The value of P is obtained directly from Thompson's tables of the 4-variable psi-function (12), available in the Unpublished Mathematical Tables File of *Mathematical Tables and Other Aids to Computation.*

III) are similarly compared with those of the immunity trial, the difference is still significant (*i.e.*, $P < 0.025$). Analysis of the total number of induced illnesses among the 18 men in whom the infectivity of the inoculum was tested subsequent to the immunity trial in the same or lower dose (Column IV) gives a value of P of 0.0037. As a matter of interest P is also given for results obtained with a 7-ml. dose before the immunity trial was attempted (Column V).

These data are sufficient to show that the failure of the five men to suffer a second bout of Marcy gastroenteritis when they were challenged approximately a year after recovery from an initial attack was not due to lack of infectivity of the inoculum. Since experimental Marcy gastroenteritis is known to confer active immunity (1, 4–6), it can be deduced that immunity lasted during the interval between inoculations. No assumptions regarding the stability of the Marcy agent during prolonged storage need be made to support this conclusion. The findings are in accord, however, with the supposition that the potency of the inoculum was about the same throughout the entire period in which it was employed (Table I), although there can be no direct demonstration.

DISCUSSION

The most convincing method of showing active immunity to an infectious agent is to challenge recovered subjects with potent inocula. The number of subjects needed depends upon the attack rates in the experimental and control groups. In this investigation only five recovered volunteers could be brought together for reinoculation. Since none developed gastroenteritis after challenge with a dose of fecal supernate capable of infecting most of the volunteers exposed to it for the first time, however, it is logical to conclude that the experimental disease suffered by the five men approximately a year before had conferred substantial immunity that persisted during the interim.

This conclusion is at odds with the tentative interpretation of a previous test of duration of immunity in two other men (5). It was stated then that two subjects reinoculated approximately 10 months after an induced attack experienced a second bout of the disease, and it was suggested that immunity had diminished during that time. While the conflicting results may be due to the variation in the immune response of individuals to be expected in this as in other infections, re-examination of the clinical records of these two volunteers leads to another explanation. In the light of experience with the experimental disease gained since then, it is felt that one of the two men may not have had a definite illness following his first inoculation; the other one did, but displayed only borderline signs after reinoculation. The criteria for diagnosis in the present study were more strict, and no such doubts cloud the clinical findings on which the analyses presented here are founded.

Excluding these two questionable cases, a total of 21 persons recovered from a prior experimental illness have now been reinoculated with the Marcy agent and all were resistant to reinfection. Kojima and his co-workers (13) had almost identical results with a strain of afebrile nonbacterial gastroenteritis from Niigata Prefecture, Japan; each of 22 recovered volunteers whose immunity was similarly checked remained well following reinoculation. In the United States it has not yet been possible to carry out reciprocal tests of active immunity with two or more different strains of the afebrile type, as has been done with the afebrile and febrile types (1). Recent Japanese experiments indicate that infection with the Marcy strain may give immunity to one of the Japanese strains (14), but the full results are not available.

This study was conducted with a limited number of volunteers at a time when little inoculum known to be safe for human experimentation was on hand. It was hoped that by combining appropriate experiments a maximum amount of information about immunity in Marcy gastroenteritis could be gathered. This was accordingly done when men volunteered and opportunities arose, and was the reason that serum was mixed with the fecal inoculum for Inoculations 8 and 9 (Table I). Kojima and his co-workers (13) had failed to demonstrate neutralization of their Niigata strain by 2- to 3-week convalescent-phase sera from recovered subjects, and it was suspected that this might also be the case with the Marcy strain. For Inoculations 8 and 9 of our study, vials of supernate were withdrawn from the dry ice box, thawed, pooled, and mixed. To an appropriate volume of pooled supernate was added an equal volume of pooled acute-phase or convalescent-

phase serum respectively. The mixtures were held in an ice bath for an hour; it was feared that at a higher temperature the Marcy agent might be inactivated, experience having been restricted to experiments in which the inocula were kept cold. Under these conditions the results were like those of Kojima and his co-workers (13); there was no apparent neutralization of the Marcy agent by either pool. The information is of limited value since antigen-antibody combination may not have taken place at the temperature of melting ice, or if it did, dissociation of the complex may have ensued in the gastrointestinal tract. The additional procedure did not interfere, however, with use of the results to determine potency of the inoculum.

At present neutralization tests in orally inoculated volunteers seem impracticable for demonstrating humoral immunity or relating the incitant of a nonbacterial gastroenteritis outbreak to either the Marcy or the Niigata agents. Attempts to detect Marcy antigen in complement-fixation tests with treated fecal supernates or inoculated tissue cultures have also been unproductive (15). So far the incitants of nonbacterial gastroenteritis can be studied only in connection with clinical illness of humans and investigation is hampered by restriction to transmission experiments in volunteers.

The data presented here suggest that the agents, presumably viruses, that may be responsible for the periodic recurrences of diarrheal nonbacterial gastroenteritis that have been observed in the Northeastern United States (2, 3, 7, 8) are diverse. Whether different outbreaks are caused by unrelated viruses, by a family of viruses, or by antigenically different strains of a single virus is a subject for future investigation. The results of this study may be useful in approaching the problem.

SUMMARY

A study was made of the persistence of immunity in five human volunteers following infection induced by oral inoculation of the Marcy strain of afebrile nonbacterial gastroenteritis. Active immunity was tested by feeding an aliquot of an inoculum demonstrated to be highly infectious. Of the five recovered volunteers, four were brought together for the reinoculation experiment 15 months after onset of the initial experimental illness; the fifth had experienced his induced attack 9-½ months before. None of the five had a second attack, indicating that immunity had persisted during the interim. The results support the possibility that yearly recurrences of nonbacterial diarrheal disease may be due to separate nonbacterial agents, presumably viruses, or to antigenically different strains of the same agent.

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UNESTERIFIED FATTY ACID IN HUMAN BLOOD PLASMA [1]

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The existence of small quantities of unesterified fatty acid in blood plasma has been known for some years. In particular, the finding of unesterified fatty acid (hereafter abbreviated UFA) in albumin fractions prepared from fresh human plasma by the methods of Cohn, Hughes, and Weare (1), and the report of Davis (2) that fresh serum yielded small quantities of UFA, indicate that UFA actually exists as such in plasma and is not a laboratory artifact. In connection with the investigations into the mechanism of lipid transport being carried forward in this laboratory, it had been found (3, 4) that a principal product of lipemia clearing *in vitro* is unesterified fatty acid. Study of the relationship of physiologic clearing *in vivo* to the concentration of circulating UFA therefore suggested itself, and led to the present investigation.

It will be observed that the term "unesterified fatty acid" is used in preference to "free fatty acid" in spite of the widespread acceptance of the latter in fat and oil chemistry. The choice is due to the evidence (5–7) that higher fatty acids are firmly, though reversibly, bound to serum albumin and at times to other plasma proteins when proteins and fatty acids exist together in aqueous solution. The properties of the fatty acid ions, such as surface activity, are therefore not in evidence when small quantities of these substances are present in serum or plasma (8). We suggest that the term "free" be reserved to describe the fraction of unesterified fatty acid not bound to protein, a fraction which is not measurable by present techniques, but which may later prove to be of biochemical significance.

[1] A preliminary report of this material was given at the Gordon Conference of the American Association for the Advancement of Science, Meriden, N. H., June 14, 1955. The subject matter also coincides in part with material being submitted as a thesis to the Department of Biochemistry, Georgetown University, by Miss Cherkes, in partial fulfillment of the requirements for the degree of Master of Science.

MATERIALS AND METHODS

For this initial investigation, the procedure of Davis (2) for the determination of UFA in blood plasma was employed, with modifications as follows: Blood samples were taken from antecubital veins except where otherwise noted (a tourniquet being applied briefly), treated with solid sodium oxalate 1.5 mg. per ml. to prevent clotting, and immediately chilled in ice water. Centrifugation was carried out in the cold, and the extract prepared within four hours. These precautions were intended to reduce *in vitro* lipolytic action which might cause artifactual elevation of the UFA concentration. Cold storage of normal or lipemic plasma samples for periods of up to four hours has been shown not to result in significant changes of UFA concentrations. To 2 ml. of plasma were added, in order, 1 ml. phosphate buffer of pH 6.0 and molarity 0.2, 1 ml. 5 per cent sodium dodecyl sulfate, 9 ml. saturated aqueous sodium sulfate, and finally a slight excess of anhydrous sodium sulfate powder. The reagents utilized were of the best commercial grade. Five extractions with 2-ml. portions of ethyl ether were carried out as described by Davis. After gentle evaporation of the ether, the residue was taken up in 5 ml. 95 per cent alcohol, a drop of approximately .01 normal sulfuric acid was added, and the solution titrated. The titrant was .02 normal sodium hydroxide (aqueous solution) delivered from a Gilmont ultramicroburette. CO_2-free air served to stir the solution, and the titration was followed with a Beckman Model G glass electrode pH meter. Titration between the apparent pH limits of 6 and 10 was found to give excellent agreement with the known molarities of standard higher fatty acids. After deduction of an appropriate blank, the concentration of UFA in an unknown plasma could be calculated in milliequivalents per liter. The data have not been corrected for the small change in plasma volume due to addition of sodium oxalate. In the course of the work it was noted that the dried fatty acid extracts, when stored in the refrigerator, were quite stable in terms of their acid equivalence. Not more than 24 hours was permitted to elapse, however, between the preparation and titration of the extracts with which this report is concerned.

The human subjects studied in this investigation are from two sources, inpatients at the Clinical Center of the National Institutes of Health (the normals in this group being primarily conscientious objectors), and a number of laboratory employees who had been found healthy in a routine pre-employment medical work-up, and who were free of acute disease on the day of study.

TABLE I

Recovery of fatty acids from plasma

Acid	Amount added *mEq./L.*	Recovery %
Stearic	0.91	99, 103
Palmitic	0.90	96, 97
Myristic	0.89	98, 103
Lauric	0.84	99, 102
Capric	0.93	101, 96
Caprylic	0.99	85, 87
Caproic	1.33	33, 32
Butyric	0.63	2, 8
Propionic	1.15	Less than 1
Linoleic	1.08	80, 82
Linolenic	1.06	84, 85
Oleic	0.80	98, 98

OBSERVATIONS

It was deemed necessary initially to confirm the validity of the method of analysis; Davis' original paper (*2*) does not provide proof that the procedure is quantitatively accurate. Recovery experiments were therefore carried out by adding small amounts of fatty acids to a plasma whose UFA had already been determined. The results of this study are shown in Table I. The recovery of all the higher fatty acids was excellent with the exception of the polyunsaturated compounds, with which a loss of approximately twenty per cent occurred. This loss, even if not due to contamination of the acid samples with foreign acidic substances, is felt to be too small to detract appreciably from the studies which follow.

Of the substances which might be expected to interfere, all but two could be eliminated from consideration. Some phospholipids have a titratable acid group; their presence in the extract might be expected to lead to inaccuracies. Extracts prepared as described from normal plasmas, however, were consistently found to contain only negligible traces of lipid phosphorus. A number of carboxylic acid anions were added to plasmas in recovery experiments. Of these, oxalate, citrate, ethylenediaminetetraacetate, ascorbate, urate, lactate, pyruvate, and acetoacetate were found not to interfere. Beta-hydroxybutyrate behaved like butyrate, with 7 per cent of the added material being recovered, whereas in the case of cholate, a recovery of 23 per cent was noted. In each case, the per cent recovered was independent of the amount added over a wide range of concentrations. It therefore appears that these two acids, which are so similar to the fatty acids in their solubility and titration properties, may contribute somewhat to the UFA value as determined by this procedure. The contribution must be a minor one, however, unless the concentrations of these substances which have been reported in human blood plasma are grossly underestimated.

In a considerable number of instances, analyses by this method on normal plasma samples taken under various conditions were performed in duplicate. Statistical analysis of these data reveals that the method itself has an intrinsic standard error of 0.061 mEq. per L. (exclusive, of course, of possible systematic errors). Because the biologic variations encountered in the subsequent study so greatly exceeded the variance of the analytical method, it was deemed advisable not to attempt to do all analyses in duplicate, but rather to increase the number of subjects studied. For the same reason, in the study of the effect of various stimuli on the UFA concentration, the technique of following serial samples in each subject, and so of using each individual as his own control, was adopted. The data will be presented in graphic form so that the course of each subject can be followed.

The first study to be undertaken was an investigation of the effect of oral fat feeding on the UFA concentration. Subjects for this study were on a normal diet; they ate no food after supper on the day preceding the test. After a fasting blood specimen had been obtained, the subjects ingested a palatable breakfast containing 100 gm. fat (sup-

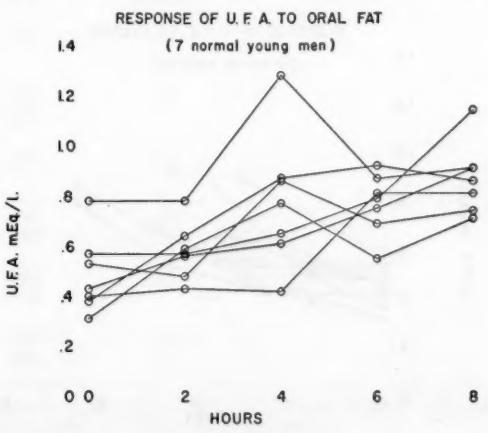

FIGURE 1

plied in eggs, bacon, cream, and oleomargarine), 40 gm. protein, and negligible carbohydrate. Samples were taken at two-hour intervals for eight hours, the subjects having no further nourishment during the day. To complement the UFA determinations, total lipid analyses were performed according to the method of Bragdon (9). The effect of the fat feeding on the UFA values is shown in Figure 1. It will be noted that in only three out of seven instances was there a rise in UFA concentration followed by a fall, and in each of these the final UFA level appreciably exceeded the initial value. When the UFA and total lipid values for each individual were compared, it was found that the peak of lipemia, usually at four hours, did not necessarily coincide with the highest UFA value, but rather that UFA often continued to rise after clearing of lactescence and of chemically measured lipemia was complete.

For comparison with the effect of oral fat feeding, the effect of fasting was studied in normal young subjects, many of them the same individuals that had been used in the fat-feeding studies. The procedure was identical, except that the breakfast was omitted, and the fast was terminated after 4 or 6 hours of observation (18 to 22 hours' total fasting). The data are presented in Figure 2. Of course no lipemia was present, but the UFA values fall in the same range as those observed after fat feeding, and showed a tendency to rise gradually as fasting continued.

The effect of feeding carbohydrate was next studied by observing the response of UFA values

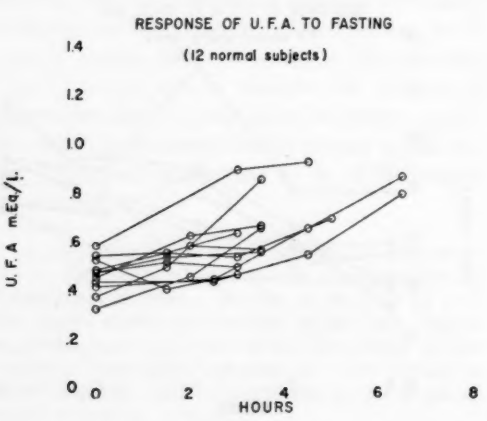

FIGURE 2

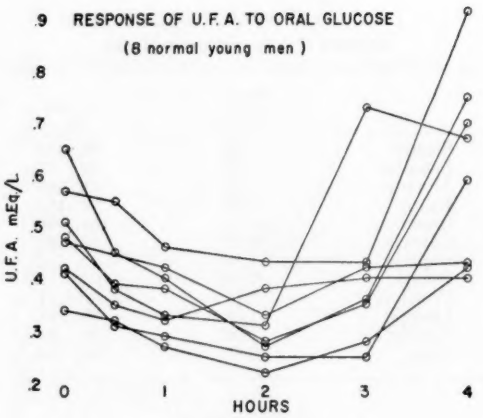

FIGURE 3

to the standard oral glucose tolerance test, a dose of 100 gm. being used. The results for eight normal young men appear in Figure 3. In addition, it was possible to obtain blood samples during glucose tolerance tests performed for other reasons on seven hospital patients, subjects who suffered from a variety of chronic illnesses not related to carbohydrate metabolism, and whose blood sugar curves were not abnormal. In each of these 15 instances, the UFA value dropped decidedly below the control level one or two hours after the glucose feeding. It should be noted in Figure 2 that spontaneous falls in UFA concentration occurred only rarely during fasting, and were never of great magnitude; the uniformity of the response to carbohydrate feeding is therefore impressive. At-

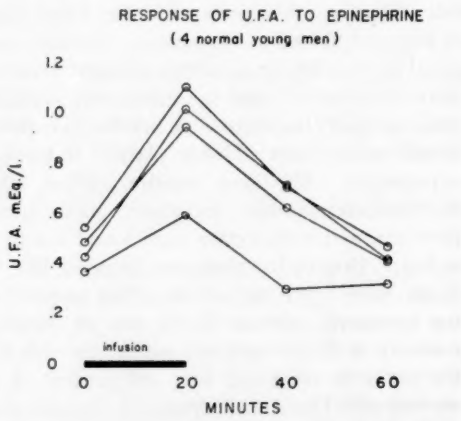

FIGURE 4

TABLE II

Extraction of unesterified fatty acids

Patient	Venous sample	Venous UFA	Simultaneous arterial UFA	A-V diff.	A-V O_2 diff.	% of O_2 to oxidize UFA
		mEq./L.	*mEq./L.*		*vol. %*	
A. E.	Coronary Sinus	0.64 0.69	0.85 0.87	0.19	10.6	70
W. V.	Coronary Sinus	0.77 0.77	0.80 0.79	0.03	14.4	7
M. L.	Coronary Sinus	0.69 0.71	0.89 0.90	0.19	Lost (Normally 10–12)	(Probably 50–60)
R. B.	Coronary Sinus	0.68 0.61	0.76 0.75	0.11	13.7	25
L. S.	Coronary Sinus	0.46 0.42	0.64 0.63	0.20	12.2	55
S. M.*	Coronary Sinus	0.76 0.75	0.94 0.98	0.20	14.8	42
S. M.* (After glucose)	Coronary Sinus	0.32 0.29	0.32 0.30	0.00	12.4	0
L. S.	Hepatic Vein	0.53 0.51	0.55 0.54	0.03	3.4	30
E. H.	Hepatic Vein	0.65 0.62	0.85 0.82	0.20	Lost	
F. V.	Hepatic Vein	0.81	0.98	0.17	7.5	70
W. H.	Internal Jugular	0.69 0.69	0.74 0.71	0.04		
J. B.	Internal Jugular	1.35 1.34	1.35 1.35	0.00		
F. F.	Pulmonary Artery	1.25 1.29	1.24 1.23	−0.03		
A. H.	Pulmonary Artery	0.99 0.97	1.05	0.07		
L. S.	Pulmonary Artery	0.61 0.64	0.64 0.61	0.00		
F. V.	Pulmonary Artery	0.99	0.95	−0.04		
F. V.	Superior Vena Cava	0.83 0.85	0.77 0.77	−0.07		
A. H.	Superior Vena Cava	0.93 0.97	0.93 0.96	0.00		
F. V.	Inferior Vena Cava	0.96 0.96	0.83 0.85	−0.12		

* INITIAL samples obtained in fasting state. With the catheter remaining in place, an infusion of 50 gm. glucose and 6 units of crystalline insulin was administered. The second samples were taken after 30 minutes.

tempts to derive a quantitative relationship between the numerical values of UFA and blood sugar were unsuccessful.

In two normal young men, an attempt was made to elicit a drop in UFA concentration by feeding glucose at the peak of a previously induced alimentary lipemia. In each case, a clear-cut drop in UFA occurred, but because of the variability of the response, it is not possible with this limited experience to compare the magnitude of the UFA decrease so induced with that occurring after feeding of glucose alone.

The sharp increase of UFA occurring in many subjects between three and four hours after glucose suggested a relationship to reactive hypoglycemia, although these subjects were not consistently those with the lowest sugar values. It therefore appeared desirable to investigate the effects on UFA of hormones concerned with blood sugar regulation. The response of UFA values to the infusion of 1 milligram of epinephrine intravenously over a twenty-minute period has been followed in four normal young men, the results being shown in Figure 4. It is evident that epinephrine administration resulted in a considerable, but short-lived elevation of the circulating UFA level. Two subjects were tested with the same dose administered subcutaneously with similar, but less dramatic, results. Other hormones affecting glucose metabolism are to be tested in the future.

The work of Lever, Smith, and Hurley (10, 11) and of Herbst and Hurley (12) leaves little doubt that intravenous administration of heparin in the presence of lipemia liberates considerable quantities of unesterified fatty acid, though their results may be due in part to *in vitro* lipolysis occurring in the samples prior to analysis. Grossman, Palm, Becker, and Moeller (13) have reported also that heparin injection in rats causes an increase in petroleum ether-soluble acid, which they believe to represent UFA. It appeared desirable to recheck this observation in humans with the present method, using the precautions which have been enumerated to minimize, if not to eliminate, *in vitro* lipolysis. This procedure was carried out in eight subjects, four of whom suffered from idiopathic hyperlipemia. The doses of heparin ranged from 10 to 50 milligrams. In every case but one, a normal who had fasted overnight, there was a significant increase in UFA ten minutes after heparin injection.

As will be explained subsequently, the foregoing observations suggested that arteriovenous differences in UFA concentration might be discovered if sought. Specimens of blood from various parts of the human vascular system were therefore obtained during cardiac catheterizations being carried out for the diagnosis of organic heart disease, and during measurements of cerebral blood flow. Patients undergoing these procedures were inpatients on the medical and surgical wards of the Clinical Center; the majority had some structural cardiac lesion or had had such a lesion corrected by surgery. All were fasting at the time of study, and none was critically ill, in congestive failure, or suffering from a complicating metabolic disorder. The results obtained to date are summarized in Table II, in which are tabulated arteriovenous differences of UFA encountered in various areas and, where available, the simultaneous arteriovenous oxygen difference. By assuming a molecular weight for the fatty acids, and further assuming that all the metabolically active UFA in whole blood is carried in the plasma, a calculation can be made of the percentage of the tissue oxygen uptake which would be required to effect the complete oxidation of the fatty acids simultaneously extracted. This figure, calculated for fatty acids of average molecular weight 275, is given in the last column of Table II.

DISCUSSION

These data must be taken with certain reservations due to the imperfection of the analytical method used. The main points of interest, however, are not so much the absolute values of UFA as the changes and differences. These differences cannot reasonably be attributed to interfering substances; they must reflect, at least semi-quantitatively, variations in the circulating UFA concentration.

The experimental work which has been presented indicates that the circulating concentration of unesterified fatty acid is in some way related to the nutritional state of the subject. If the energy requirements of the moment are satisfied by carbohydrate, the UFA concentration drops to a low level, whereas if they are not satisfied by in-

gested foodstuffs, the level rises progressively (at least over the period of observation, which has not been long enough to assure total depletion of hepatic glycogen stores). The effect of oral fat feeding on UFA concentration has not been established with certainty; our data do not suffice to demonstrate an effect of fat feeding that differs from the effect of fasting over the same period.

The rapidity with which the UFA concentration may fluctuate in an individual as his nutritional state varies implies that this may be a material of great metabolic activity. Our data appear to indicate that the concentration of UFA rises in relation to the need (in the case of epinephrine, the anticipated need) for fat as an energy-yielding substrate for oxidative catabolism. The similarity between its behavior and that of the keto acids also served to suggest that it might be directly concerned with the oxidative metabolism of lipids. The hypothesis was therefore formulated that unesterified fatty acid represents the blood lipid fraction which is immediately available to cells in general as a supply of lipid to meet energy demands, and that its release into the blood is regulated by some mechanism sensitive to the need for fat oxidation. This hypothesis led to the study of arteriovenous differences in UFA concentration, and, in turn, is considerably supported by those results.

A study of the data in Table II enables several conclusions to be drawn. There can be little doubt that the fasting myocardium extracts from the perfusing blood a considerable quantity of titratable acidity, and, if our assumption that this material is fatty acid of average molecular weight 275 is correct, the fatty acids so extracted suffice to supply the bulk of the energy requirements of the myocardium (as measured by simultaneous oxygen consumption). In the one patient in which such a study has been possible, the administration of glucose and insulin intravenously completely abolished the myocardial UFA extraction. This finding is entirely consistent with the presently accepted concept that glucose is the preferred substrate when it is available. The hepatic vein samples that have been obtained make it reasonably certain that, in addition to myocardium, the splanchnic area participates in the extraction of UFA from the blood. The two measurements of arteriovenous UFA differences in the cerebral circulation that we have had the opportunity to perform are consonant with the belief that brain tissue does not oxidize appreciable quantities of fat.

Since many tissues appear to extract UFA from the blood, it is necessary that the material be released into the blood elsewhere in order to maintain an arterial level. Lung would not seem likely as a source of UFA, and the pertinent measurements to date indicate that lung neither adds nor extracts an appreciable amount of UFA. The source would appear to be peripheral in view of the finding of superior and inferior vena caval UFA levels which exceeded the arterial concentrations. It is entirely reasonable to suppose, on the basis of present evidence, that adipose tissue is the source of the circulating UFA, since it contains the fat stores which must be mobilized and consumed during prolonged fasting. In the light of the recent demonstration by Korn and Quigley (14) of the existence of lipoprotein lipase (clearing factor) in the adipose tissue of rats, and their as yet unpublished preliminary evidence that the same or a similar enzyme exists in human adipose tissue, the supposition becomes even more attractive.

The question with which the investigation began, then, can be answered only tentatively at this time. Circulating UFA concentrations may well reflect the activity of lipoprotein lipase located in tissues and releasing lipid into the general circulation in this form for catabolism. Whether or not the process of clearing an alimentary lipemia contributes to the circulating UFA concentration remains in doubt, but it seems clear that the concentration cannot be taken as an index of the activity of the clearing process, since administration of glucose, which does not prevent the clearing of lipemia, causes a sharp drop in the UFA level. It is also apparent that high levels of circulating UFA may occur under conditions in which lipemia is minimal, and the clearing process inactive.

SUMMARY

The concentration of unesterified fatty acid (UFA) has been measured in human blood plasma by the method of Davis, which has been found to be approximately quantitative for this purpose. The UFA values fluctuate rapidly and markedly

from time to time in normal individuals. The values tend to rise after ingestion of fat, but rise similarly on fasting, so that lipemia *per se* cannot be shown to be correlated with increase in UFA. Carbohydrate feeding produces a uniform fall, whereas epinephrine injection causes an increase. The hypothesis is offered that UFA is the blood lipid fraction primarily concerned with the supply of fats to tissues for oxidative metabolism. The extraction of this substance by tissues has been demonstrated, and evidence suggesting its origin in adipose tissue obtained.

ACKNOWLEDGMENTS

The authors are indebted to Dr. Joseph Bragdon for the analyses for total lipid and lipid phosphorus, and to Mr. Fred Bullock for oxygen content determinations. We also wish to thank the many members of the Staff of the Clinical Center who made patients available to us for study and particularly those who assisted in obtaining blood specimens.

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VIRILIZING ADRENAL HYPERPLASIA; A GENETIC AND HORMONAL STUDY[1]

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It is not at all unusual to find other affected children among the brothers and sisters of patients with virilizing adrenal hyperplasia. Such familial aggregations of affected children suggest, at least, a genetic basis for the condition at hand, and when the affected individuals are distributed in one generation only, there is a possibility that the condition is due to a mutant gene which finds obvious expression in the homozygote. This mode of inheritance for a possible gene responsible for virilizing adrenal hyperplasia has been suggested (1–3). Knudson (2) obtained information on 8 families, including 11 affected children. To these he added 26 families taken from the literature in which there were 32 affected children. Comparison of the observed number of affected sibs of index cases with that expected indicated sufficient agreement to favor the hypothesis of a recessive gene. All of Knudson's cases, however, were patients who showed evidence of electrolyte disturbance, and there appears to be no study which includes all types of cases which fall into this syndrome. This paper consists of information pertaining to a possible genetic etiology of this disease, obtained from 56 families containing one or more affected individuals, and an hormonal study of a group of parents of affected children. An exhaustive review of the literature is not relevant here, since our interpretations are based only upon the results of the analysis of our own data and not upon information from families that has been presented in other articles.

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MATERIALS AND METHODS

The data were obtained from the families of all patients with virilizing adrenal hyperplasia who were examined and diagnosed in the endocrine clinic of the Harriet Lane Home since 1945. Diagnostic criteria are those given by Wilkins (4). In each instance the information was given by the mother or responsible parent, and in a few cases by the patient himself. Some questions were put by means of a questionnaire which was mailed to all families, but since in some cases parents failed to answer the questionnaire and were otherwise unavailable, the data are in certain aspects incomplete.

The data consist of information pertaining to: disease in sibs and other relatives, together with intra-family characteristics of the disease; age of sibs, their sex, maternal age at birth, birth order, death, age, cause, and place of death; maternal gestation and parturition; parental consanguinity; and size of the families of both parents.

In some families all affected children were patients of the endocrine clinic, but there were 12 sibs of endocrine clinic patients who are presumed to have or have had the disease, who have never been seen in the Harriet Lane Home. Of these, 3 are still living, and detailed inquiry reveals that a firm diagnosis has been made on all three. The remaining 9 were dead by the time the index case was seen here. A diagnosis, either during life or at autopsy, was made elsewhere on 5 of these. This leaves 4 cases who were dead and who were not diagnosed as patients with virilizing adrenal hyperplasia either during life or at death. The history of these 4 children was, however, compatible with the disease. All were males without genital abnormalities, who though normal at birth failed to thrive and died in the early weeks of life with dehydration, diarrhea, and vomiting, often accompanied by pneumonia. Sibs dead of unknown causes were excluded.

Measurement of urinary 17-ketosteroids, pregnanetriol, and pregnanediol

Urinary 17-ketosteroids were determined by a modification of the method of Callow, Callow, and Emmons (5). Fifty-ml. aliquots of freshly collected 24-hour urine specimens from 18 normal adult male and female subjects and 20 parents of patients with virilizing adrenal hyperplasia were incubated with a beef liver preparation of beta-glucuronidase (Ketodase, Warner-Chilcott Laboratories)

for 24 hours at 37°C after adjusting the pH to 4.5 with 2 N acetate buffer, and the hydrolysate was extracted with benzene. The benzene extract was washed with 1.0 N sodium hydroxide and distilled water, evaporated, and the residue dissolved in 5.0 ml. of benzene with gentle heat, applied to an alumina (Harshaw Chemical Co.) column, and chromatographed according to the method of Bongiovanni and Clayton (6) as devised in this laboratory. No preliminary Girard separation was performed. The 2 per cent ethanol in benzene eluate was used to determine pregnanediol chromogen, and the 10 per cent ethanol in benezene fraction was employed to determine pregnanetriol chromogen. Both fractions were dried with a stream of air, the residue redissolved in 5.0 ml. of ethanol to complete removal of any residual benzene, and again evaporated to dryness. Pregnanediol and pregnanetriol chromogens were obtained by adding 10.0 ml. of sulfuric acid (A.R.) to the dried residue, and the chromogen formed was read after 20 minutes with the Beckman D.U. quartz spectrophotometer at the following wave lengths: 325, 390, 425, 440, 460 mu. The total quantity of each substance was calculated by the use of the corrected coefficient of extinction at 425 mu, as previously described (6). For pregnanetriol to be considered present, the sulfuric acid chromogen of the unknown was required to conform to the spectrum of pure pregnanetriol in sulfuric acid. The rapid addition of water to the sulfuric acid chromogen of pregnanetriol consistently produced a transient pink color as observed by Bongiovanni, Eberlein, and Cara (7). In addition, a comparison of acetaldehydogenic substances in the residue of the 10.0 per cent ethanol in benzene eluate after periodate oxidation according to the method of Cox (8) has shown good agreement in this laboratory with the determination of pregnanetriol by the chromogen formed with sulfuric acid: the former measures C 17-, 20-dihydroxy, 21-methyl steroids. Furthermore, as pointed out previously (6, 7), the application of this method has resulted in the isolation of crystalline pregnane-3α, 17α, 20α triol, the identity of which has been confirmed by infra-red spectroscopy.

ACTHAR gel,[5] 40 I.U. per ml., was administered intramuscularly between 8 A.M. and 9 P.M. in a dosage of 60–80 I.U. daily for one to two days. The group of normal adults all received at least 80 I.U. daily, while in several instances parents of affected individuals received 60 I.U. in a single dose. Urine was collected the day prior to administering the ACTH and during the 24-hour period after each injection of ACTH. The urine was preserved with 5 ml. of toluene.

RESULTS

There were 76 affected individuals among 181 sibs in 56 families. There were no affected parents, grandparents, uncles, aunts, or cousins. Among genetic hypotheses this distribution suggests a gene which manifests itself only in the homozygote. Alternatively the disease might not be genetically determined at all, but might be due to an environmental effect, possibly an effect of the maternal, intra-uterine environment. These two possibilities we have examined in detail.

It is perhaps unlikely that this condition could be due to an effect of the maternal environment for the following reasons: 1) These patients have a metabolic defect which is operative throughout life, and sometimes showing no clinical manifestations until after birth, whereas defects due to intra-uterine factors are fixed at the time of birth and do not change, except as growth and development may alter and distort their effects. 2) Female pseudohermaphrodites born to mothers with arrhenoblastoma show no additional virilization after birth (9). 3) Genital defects produced in the offspring of animals to whom androgens have been administered during pregnancy may be similar to those exhibited by patients with virilizing adrenal hyperplasia, but these are fixed at birth, and no further virilization ensues (10, 11). 4) There is a striking similarity between the proposed pathogenesis of this disease (6, 12–17), and biochemical defects in a variety of organisms for which there is excellent evidence for gene control.

These reasons do not rule out this possibility, but make it somewhat less satisfying than the alternative possibility of genetic control. An analysis of the data for the operation of certain maternal factors will be given later in the paper.

Evidence favoring the hypothesis of a single, autosomal, recessive gene depends upon the fulfillment of several criteria. We have used those given by Penrose (18). If this disease were due to such a gene we should find: clear distinction between affected and unaffected; a ratio of one affected to 3 healthy offspring of unaffected parents, boys and girls showing the disease with equal frequency; if the disease is uncommon, a higher incidence of first cousin matings among the parents of affected children than among the general population, and occasional cases appearing among the collateral relatives.

Distribution of affected within families

A glance at Table I reveals that considerably more than one quarter of the children are affected.

[5] Lot No. N-33107. We are indebted to Dr. J. A. Hubata and the Armour Company for contributing this preparation.

TABLE I

Factorial method of testing recessive hypothesis on families containing cases of virilizing adrenal hyperplasia

Size of sibship S	No. of sibships of size S N_s	Total No. of sibs $S \times N_s$	Number of affected sibs			Variance of expected number
			Observed		Expected	
			Obs.	Presumed	$S \times N_s/4[1-(3/4)^s]$	
1	8	8	8		8.00	0.000
2	16	32	18	(20)	18.28	1.956
3	14	42	22	(23)	18.16	3.682
4	9	36	13	(14)	13.17	3.780
5	3	15	4		4.92	1.776
6	2	12	2		3.65	1.552
7	1	7	1		2.02	0.970
8						
9	2	18	2		4.87	2.760
10						
11	1	11	2		2.87	1.805
Totals	56	181	72	76	75.94	18.281

S.E. $= \sqrt{18.281} = 4.28$

$$\text{Difference} = 75.95 - 72 = 3.95.$$

$$\frac{\text{Difference}}{\text{S.E.}} = \frac{3.95}{4.28} = 0.92.$$

$$\text{Difference} = 76 - 75.95 = 0.05.$$

$$\frac{\text{Difference}}{\text{S.E.}} = \frac{0.05}{4.28} = 0.01.$$

This may be due to the fact that many of the families are small, and only families which contain at least one affected child have come into the data. We may test this possibility by comparing our figures with expectation according to various family sizes, assuming the actual proportion of affected to unaffected to be precisely 1:3. Table I sets out these calculations according to a method devised by Hogben (19). It will be seen that there is good agreement between the number of observed cases and expectation, and indeed, the difference between observed and expected is less than the standard error whether we include the four presumed cases or not. In addition, the agreement within categories according to family size is good.

Haldane has devised a somewhat different method by which one may estimate the true proportion of affected children from the data, which may then be compared with the proportion to be expected (20). This calculation gives a proportion of 0.2507 with a standard error of 0.0139, a value which agrees very well with the expected proportion of 0.25.

These calculations do not of themselves prove that adrenal hyperplasia is due to a recessive gene, but do indicate that the distribution of affected children in the families in the data does not differ significantly from that expected if the disease were due to such a gene.

In addition here is further evidence against a maternal factor, since if a maternal factor were causing the defect, we would not expect such good agreement between observation and expectation within each family size category.

Gene frequency

Assuming for the moment that virilizing adrenal hyperplasia is genetically determined, it is relevant to the analysis of our data to estimate the gene frequency. This estimate will necessarily be imprecise, but nevertheless useful.

To do this we must know the incidence of the disease, a figure difficult to find, particularly if the disease is rare, or affects viability. For example, some cases of adrenal hyperplasia would go unrecognized through neonatal death, or through ignorance of the parents, and some males with delayed virilization might be regarded as instances of precocious puberty and thus be lost. In addition, any one physician or institution will have no more than a sample, be it large or small, of the total.

The possibility of making a minimum estimate in the state of Maryland is perhaps favorable, since circumstances make it likely that the ma-

jority of patients who come to the attention of physicians at all, will find their way to the endocrine clinic at the Harriet Lane. A rough incidence may therefore be calculated by comparing the number of affected patients born in Maryland with the number of births in the state since 1939,[6] the year 1939 being chosen because the eldest child in the group was born in that year. There were twelve patients born in Maryland during this interval, against 798,118 live births in the state. This gives an estimate of the incidence of the disease in Maryland of one per 67,000 births. It is of interest that but one colored patient, appeared in the entire series. This is doubtless due in part to social factors, but may also represent a different incidence of the disease among negroes.

We may now estimate the gene frequency by means of the Hardy-Weinberg Law, obtaining a value of 0.0039 and an incidence of heterozygotes of 0.0078 or one per 128 individuals in the general population. It should be emphasized that these estimates are crude, and that the true values are certainly higher than these. But the calculations are perhaps worthwhile in indicating that though the disease may be rare, the presumed gene would exist in rather large numbers in heterozygotes in the population.

Consanguinity

In our series there was only one family, in which the parents were related in any way, and these were first cousins. The finding of one consanguineous union in this series could be fortuitous, but it is possible to calculate how many such matings one would expect, using the gene frequency value we have already estimated.

This calculation (Dahlberg [21]) requires an estimate of 1st cousin marriages in the general population, and for this figure we have used the incidence quoted by Glass (22) of one per 2000 marriages, based upon interviews with 8000 Maryland families. The calculation reveals an expectation of one first cousin marriage per 120 families, a ratio considerably higher than that in the general population. We have found one in 47 families. Thus we may take it that we would not expect more than one consanguineous mating among our families, but it is not surprising that one does appear. Consanguinity has been reported in a number of families reported in the literature (2, 3).

Collateral relatives

We did not find any affected individuals among the uncles, aunts, and cousins. The disease is obviously rare, and even if the gene frequency were 2 or 3 times as great as our estimate, the incidence of the disease among collateral relatives would be so small as to be unexpected in 56 families.

Sex ratio

If the proposed gene is an autosomal recessive it ought to be distributed equally among males and females. The data, however, reveal a heavy preponderance of females. One possibility is that the surplus of females might be due to biases in the ascertainment of cases, and the data were therefore examined with this possibility in mind. The proportion of males among all cases was 0.43. Considering only the index case in each family the deficiency of males is even greater (0.33), suggesting that females may be more readily ascertained than males, probably because of genital abnormalities as well as the striking contrast between their sex and the evidence of virilism. On the other hand, some families containing only males who may show less striking abnormalities, might not enter a series such as this because of failure to recognize them.

If, on the other hand, both males and females are equally prone to the electrolyte imbalance seen early in life in this disease, we might see a more equal distribution of the sexes among these patients. In such cases a true diagnosis is more likely to be made, because of the urgent need for correct diagnosis and treatment of the disease at this stage. In our data, a comparison of males and females among salt losers reveals a proportion of 0.65 males. The bias of ascertainment may be eliminated altogether if we consider only those patients in our families who were discovered after a diagnosis had been made on the index case. There were twenty of these, 9 males and 11 females. This ratio does not depart markedly from unity, and suggests that males are affected equally with females. This information is summarized in Table II.

[6] These figures were kindly furnished by Dr. Edward Davens of the Maryland State Health Department.

TABLE II

Ratio of males to females among patients with virilizing adrenal hyperplasia

	Males	Females	Totals	Sex-ratio M/F
Index cases	14	42	56	0.33
All cases	23	53	76	0.43
Salt losers	11	17	28	0.65
Affected sibs	9	11	20	0.82

Clinical heterogeneity

It is well known that virilizing adrenal hyperplasia shows a varied clinical picture. In addition to virilism some patients show electrolyte disturbances, and some are hypertensive. There is also some variation in age of onset. That is, some females exhibit enlargement of the clitoris and urogenital sinus at birth, others have only clitoral enlargement, and a few are quite normal at birth and show delayed onset of virilization. In boys, signs of the disease may consist only of electrolyte disturbance in the neonate, or the onset of virilism may be delayed for a variable length of time.

It was a matter of some interest to examine the data to see whether this heterogeneity existed between or within families. There were 16 families that contained at least one affected child who showed electrolyte imbalance. Of these, 9 families had two affected children, both of whom were salt losers, and in one family there were 3 such patients. There were no families containing more than one affected child in which one was a salt loser and others were not.

Three families contained at least one affected child who was hypertensive. Of these, two contained two patients, both hypertensive. None of these patients were also salt losers.

There were four families in which affected females had either normal genitalia at birth, or some degree of clitoral enlargement without a urogenital sinus. In 3 of these there were female sibs who showed a similar picture at birth. Two pairs of these girls are also hypertensive.

In one family there were two boys, the onset of whose disease was delayed until each was about 6 years. Table III summarizes this information for 18 families which contained two or more affected children.

Thus it is clear that in this series the heterogeneity of the clinical picture of the disease appears between families, but not within. In each family the manifestations were always the same in affected sibs, salt loss and hypertension did not occur together, and in none of the cases in which the onset was delayed was an electrolyte disturbance found. For this kind of analysis the numbers of cases are small, so that some or all of these conclusions may be erroneous. Confirmation or rejection will have to await the analysis of additional data.

Maternal factors and family size

It is pertinent to examine the effects of genes on the fertility of their carriers. If adrenal hyperplasia is genetically determined, and present only in homozygotes, then both parents must be heterozygous. The question arises whether a gene which in the homozygote renders the victim sterile, might have some effect upon the ability of the heterozygous mother to conceive and bear children.

Pregnancy losses of 51 mothers were investigated and found to amount to 9.94 per cent of all pregnancies. There were perhaps other early miscarriages unknown to these mothers, but this rate is certainly not extraordinary (23).

In addition we have looked for evidences of effects which might be expressed in the gestational characteristics of the mother, such that maternal age or parity might influence the manifestation of the disease in her offspring. This information consists of a comparison of numbers of offspring observed at various birth ranks and maternal ages at births of affected children, with expectation. The method is due to Penrose (24). Table IV shows this comparison and indicates that no noticeable effect was found. There were no diffi-

TABLE III

Indicating heterogeneity of clinical syndromes between, but homogeneity within 18 families containing more than one affected child

Type of disease	Number of index cases	Sibs			
		Salt losers	Hyper-tension	Virilism only	Totals
Salt losers	10	11	0	0	21
Hypertension	2	0	2	0	4
Virilism only	6	0	0	7	13
Totals	18	11	2	7	38

culties on the part of the mothers in achieving pregnancy, nor were there any significant histories of complications during pregnancy or labor.

Inquiry into the total numbers of offspring produced by the mothers in our series, as well as the sizes of the families whence they and the fathers came revealed that the mean family size of the fathers was 4.1 sibs, of the mothers 3.8 sibs, and of the patients, 3.2 sibs. It is evident that families containing possible carriers of the gene are not reduced in size, nor are sibships containing affected individuals, even though the latter are not in all cases completed families.

In sum, it may be said that no factors influencing conception, gestation or fertility, nor any maternal influence on the disease have been found.

Expression in possible heterozygotes

If it be assumed that every gene has a measurable effect if it could be detected, it is worthwhile to seek the manifestations in heterozygotes of genes which are expressed fully only in homozygotes. The detection of such an effect in the heterozygote in fact puts the hypothesis that the condition is heritable on much firmer ground. Since both parents of any homozygote must be heterozygous for the particular gene in question, these individuals would be the most likely ones to study for manifestations in the carrier.

Evidence has recently been presented to indicate that the primary defect in virilizing adrenal hyperplasia consists of failure of the adrenal cortex in the synthesis of compound F (12–16). In accordance with the scheme of adrenocorticosteroid biogenesis suggested by Hechter and his coworkers (25), Jailer has suggested that a block exists in the conversion of 17α hydroxyprogesterone into compound F (17). Substantiation of Jailer's hypothesis has been given by the demonstration by Bongiovanni and his co-workers that 3α- 17α- 20α-pregnanetriol is the steroid found in greatest quantity in the urine of affected patients (6, 7, 26, 27). In addition, administration of ACTH to patients with adrenal hyperplasia has caused a rise in urinary pregnanetriol, frequently of greater magnitude than the increase of 17-ketosteroids (7, 26). Pregnanetriol has also been shown to be a metabolite of 17-hydroxy-progesterone by recovery and identification of this steroid from the urine of a 10-year-old boy with Addison's disease, as well as from adrenalectomized rabbits to which 17-hydroxy-progesterone had been administered parenterally (28). Thus the synthetic transformations involving the conversion of 17-hydroxy-progesterone to compound F may well include a blocked step which could be due to a mutant gene.

This possibility suggested an approach to the detection of heterozygotes. It is clear that the heterozygote performs adequately the synthesis which is blocked in the homozygote, but excessive stimulation of the adrenal cortex of the carrier might stress this pathway of synthesis so as to reveal a normally concealed defect. This might result in abnormal increase in urinary pregnanetriol.

Measurements were therefore made of pregnanetriol in the urine of 20 parents before and after stimulation with ACTH, in order to determine whether or not a rise in this metabolite would

TABLE IV

Comparison of observed and expected numbers of patients with virilizing adrenal hyperplasia according to birth rank and maternal age

A. Birth Rank

	1	2	3	4	5	6	7	8	9	10	11
Observed	22	20	14	7	2	1	0	0	0	1	0
Expected	23.89	20.46	11.96	5.76	2.03	1.12	0.62	0.29	0.29	0.29	0.18
√Variance	2.79	2.87	2.38	1.74	1.06	0.71	0.56	0.41	0.41	0.28	0.28
Total obs.	67										
Total exp.	66.99										

B. Maternal Age

	15–19	20–24	25–29	30–34	35–39	40–44	Totals
Observed	4	14	19	9	3	1	50
Expected	3.44	15.50	18.72	8.84	2.70	0.75	49.95
√Variance	1.03	2.03	2.41	1.94	1.16	0.61	

TABLE V

Twenty-four hour excretion of pregnanetriol of 18 normal controls and 20 parents of patients with virilizing adrenal hyperplasia

Controls					Parents				
		Pregnanetriol *mg./day*					Pregnanetriol *mg./day*		
Sex	ACTH	Initial	Post ACTH	Increment	Sex	ACTH	Initial	Post ACTH	Increment
M	80	1.6	3.1	1.5	F†	80	0.85	10.5	9.7
M	80	1.2	2.0	0.8	M	80	0.00	1.2	1.2
F†	80	1.2	5.6	4.4	F	80	0.44	3.9	3.5
M	80	1.5	2.0	0.5	M	80	1.10	2.3	1.2
F	80	1.3	3.1	1.8	F	60	1.40	3.9	2.5
M	80	2.6	3.4	0.8	M	60	0.95	2.1	1.1
M	80	3.1	3.3	0.2	F	80	0.57	2.8	1.7
M	80	1.6	1.6	0.0	M	80	1.30	5.0	3.7
F	80	2.0	2.4	0.4	F	60	0.52	1.6	1.1
F	80	0.4	1.0	0.6	M	60	1.20	2.4	1.2
F	80	1.4	1.9	0.5	F	80	0.66	1.9	1.2
F	80	0.5	1.8	1.3	F	60	1.10	1.9	0.8
M	80	1.3	1.7	0.4	M	60	0.81	1.3	0.4
F	80	1.8	3.4	1.6	F	80	0.75	2.9	2.2
M	80	1.5	2.6	1.1	M	80	0.48	2.1	1.6
M	80	1.0	2.1	1.1	F	80	3.70	6.3	2.6
F	80	1.7	2.6	0.9	M	80	1.40	2.1	0.7
F	80	2.6	4.1	1.5	M	80	5.60	46.9	41.3*
					M	80	1.6	2.8	1.2
					F	80	0.27	0.9	0.5
Means and S.E. of Means		1.59	2.65	1.06±0.30			1.01	3.05	2.04±0.47

* Not included in mean.
† Pregnant.

appear. In addition, 17-ketosteroid and pregnanediol determinations were run on the same specimens. As controls, 18 normal, unrelated individuals, both male and female, in a sex distribution approximately equal to that of the parent group were stimulated in the same manner and the same determinations were made. Table V shows the results among both controls, and parents. It should be noted that one extremely high parent value has not been included in computing the mean. This parent showed an increment of 41.3 mg. per day. This value was checked by a second collection of urine following a second dose of ACTH, and on this occasion the pregnanetriol excretion was 43.9 mg., representing an increment of 38.3 mg.

A test of significance of the difference between the increments of parents and controls gave a p value lying between 0.02 and 0.05, suggesting that the difference was a significant one (29). Thus we may say that though the number of our observations is small, the difference in increments between the two groups is significant, although not highly significant.

When frequency distributions of the two sets of values were drawn up, though there was much overlapping, much of the parent curve lay to the right of that of the controls, showing graphically

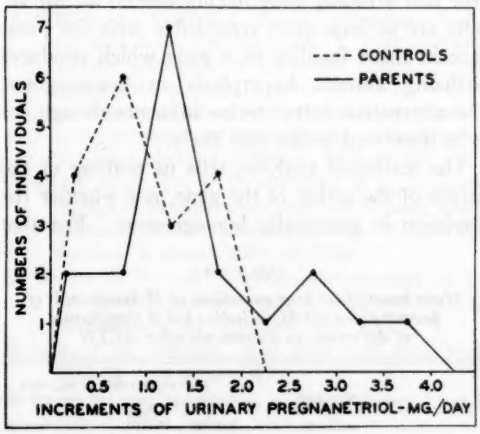

FIG. 1. FREQUENCY DISTRIBUTIONS OF INCREMENTS OF PREGNANETRIOL EXCRETION FOLLOWING ACTH STIMULATION OF 17 CONTROLS AND 18 PARENTS OF AFFECTED INDIVIDUALS

TABLE VI

Mean excretion of pregnanediol by parents and controls before and after stimulation with ACTH

	Number of subjects	Pregnanediol in *mg./day*		
		Initial	Post ACTH	Increment
Parents	13	2.61	4.72	2.12±0.63
Controls	10	3.19	5.32	2.24±1.14

the difference between the two groups. These curves are shown in Figure 1. In drawing the curves, the two highest values among the parents, and the highest value among the controls have been removed.

In Tables VI and VII are set out the mean excretion values of 17-ketosteroids and pregnanediol both before and after administration of ACTH, along with their mean increments. No significant differences were found here.

DISCUSSION

The proof of heritability of any disease among humans remains a difficult problem. It depends upon two types of evidence: a) statistical analysis of family data to determine conformity with criteria specific to particular Mendelian hypotheses, and b) observation of a biochemical abnormality which can be understood in terms of gene control.

Though the analysis of our data does not prove the presence of a heritable factor to the same degree that breeding experiments will do so, the results are at least quite compatible with the presence in these families of a gene which produces virilizing adrenal hyperplasia in homozygotes. The alternative, intra-uterine influence though not to be dismissed, seems less likely.

The statistical analysis, tells us nothing of the nature of the action of the gene, nor whether the condition is genetically homogeneous. For that information we must consider those clinical and biochemical characteristics which may be observed and measured.

TABLE VII

Mean twenty-four hour excretions of 17-ketosteroids of parents and controls, including test of significance of difference in increments after ACTH

	Number of subjects	17-Ketosteroids in *mg./day*		
		Initial	Post ACTH	Increment
Parents	20	6.68	11.82	5.14±1.54
Controls	18	9.19	11.72	2.52±1.09
t = 1.55. P = >0.1.				

It is apparent that there are variations in the clinical picture of the disease and that this heterogeneity exists between, but not within, families. More information is needed to confirm these observations, and if this is forthcoming, this heterogeneity may ultimately come to be understood in genetical terms. That is, it suggests the segregation in particular families of different genes, possibly a system of alleles at one locus, or alternatively different genes at different loci, controlling different biochemical events but producing phenotypic similarities. Evidence indicating that there may be more than one biochemical defect has been given by Bongiovanni and Eberlein who found distinctive chemical characteristics in a patient with virilizing adrenal hyperplasia and hypertension (30, 31).

As a possible manifestation of the carrier state, we have looked for evidence of increased excretion of pregnanetriol by parents of patients. The rise in excretion of this compound by parents, following stimulation with ACTH, was slightly greater than that of the controls, and differed significantly but not highly from the latter. In addition, two of the parents showed increments far in excess of any shown by the control individuals. The true meaning of these observations will have to await confirmation, but the finding does add some weight to the concept that this disease is determined by a mutant gene. It is probably worthwhile to search for other characteristics which might be more definitive manifestations of the trait. It should be emphasized that ACTH stimulation and subsequent determination of pregnanetriol excretion could not, except in an occasional instance, serve to pick out an heterozygote from the general population.

SUMMARY

1. Information obtained from 56 families which contained 76 patients with virilizing adrenal hyperplasia has been analyzed. Comparison of the observed numbers of patients with that expected upon the hypothesis of an autosomal recessive gene responsible for the condition showed good agreement. There was one consanguineous mating among 46 families, an incidence which would

be expected on the basis of a calculated minimum gene frequency. It is probable that, though there were many more affected girls than boys in the series, the preponderance of females is due to biases in ascertainment of the cases, most likely because of the abnormality of the female genitalia. The fertility of the parents and grandparents was not reduced. There were no evident deleterious effects on gestation or labor in the mothers, nor any evidence of maternal influence on manifestations of the diseases.

2. Among these cases the clinical pattern of the disease was consistent within families in which more than one case existed, but varied between families. This suggests that there may be genetic as well as clinical heterogeneity.

3. Attempts were made to detect the effects of a possible gene in the parents, presumed to be heterozygotes. Measurements were made of pregnanetriol in the urine of 20 parents and of 18 normal control individuals both before and after the administration of 60 to 80 units of ACTH. The increments in the amount of this compound excreted in the urine of the parents were compared with those of the controls. The difference was significant, at the 2 to 5 per cent level. While such a difference is not striking, it is additional suggestive evidence that virilizing adrenal hyperplasia is a genetically determined, inborn error of adrenocortical steroidogenesis.

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THE MECHANISM OF THE EXCRETION OF AMMONIA IN THE DOG

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The excretion of ammonia is dependent upon several factors: the amount and duration of acid intake, presumably affecting variations in intracellular enzyme activity (1–3), the availability of precursors from which ammonia can be formed (4–6), and the pH of the urine (7–9). With respect to the effect of urine pH, the excretion of ammonia resembles that of a number of organic bases, such as quinine (10), nicotine (11), procaine (12), Atabrine® (13), and certain derivatives of 4-aminoquinoline (13), all of which appear in the urine at a rate inversely related to urine pH. The experiments to be reported were designed to reexamine this phenomenon and to compare ammonia excretion with that of selected organic bases in order to provide information concerning 1) the nature of the process by which ammonia enters the urine, 2) the production of ammonia by the cells of the renal tubules, 3) the mechanism of excretion of other weak electrolytes, the output of which is dependent upon urine pH, and 4) to yield an interpretation of the quantitative relations between ammonia excretion and urine pH.

Since the factors which affect the transport of the organic bases are similar to those affecting the movement of ammonia into the urine, it was anticipated that the excretion of these bases might be used as a means of independently evaluating the factors affecting the transport of ammonia as contrasted with those affecting intracellular production. In acute experiments the clearance of quinine was found to serve this purpose since it was influenced by pH only and the excretion of ammonia by both urine pH and by circumstances which might be presumed to influence rate of production. However, it was not possible to extend these principles to chronic experiments since the absolute level of quinine clearance varied at the same pH in different experiments in an unpredictable fashion.

The results of the studies are in accord with the view (9, 14) that ammonia formed in the tubule cell enters the urine almost exclusively in the uncharged state, *i.e.*, as the free base NH_3, and accumulates as NH_4^+. The extent of accumulation is probably limited in the acid range of urine pH by the rate of ammonia production. At higher pH's, production of ammonia is non-limiting and the rate of excretion is dependent upon equilibration of ammonia in the tubule urine with that in tubule cell. Similar considerations also apply to the urinary loss of other weak electrolytes whose excretion is modified by changes in urine pH except insofar as rate of diffusion, rather than rate of production, is presumably the limiting factor in the acid range. The data have further been interpreted to indicate that final changes in urine volume are effected by abstraction of water in the tubule at a site beyond which final ammonia content is achieved.

METHODS

Trained, unanesthetized female dogs weighing approximately 20 kg. were used. The plan of most of the experiments was to compare the excretion of ammonia and of one or more organic bases during progressive elevation of urine pH from acid to alkaline values. In studies designed to examine the effect of urine flow on the excretion of weak bases, an attempt was made to maintain urine pH constant in either the acid or the alkaline range during the infusion of osmotic diuretics or hypotonic solutions. This goal was attained without difficulty only when the urine was strongly acid, though in the extremes of osmotic diuresis, urine pH frequently rose when it was low initially. In urines containing appreciable amounts of bicarbonate the production of diuresis lowers the concentration of bicarbonate without corresponding changes in CO_2 tension and therefore reduces urine pH. Consequently, it was impossible to maintain hydrogen ion concentration constant in the face of varying urine flow when urine pH was above 7.0.

Acidosis and some stimulation of ammonia production were induced by the oral administration of 5 to 6 grams of ammonium chloride on the day prior to most experiments. The organic bases were administered orally in

the following manner: 9-(diethylamino-l-methyl) butylamino-acridine (SN 8439, pKa 8.9),[1] 300 mg. given 12 hours before the experiment; 3-chloro-7-methoxy-9 (diethyl-amino-l-methyl) butylamino-acridine (Atabrine® pKa, 7.3), 100 to 200 mg. per day for 2 to 3 days prior to an experiment; quinine (pKa 8.3), two doses of 500 to 600 mg. each, given 15 hours and 1 to 2 hours prior to the start of an experiment, and 3-chloro-7-methoxy-9 (diethyl-amino) ethylamino-acridine (SN 5228, pKa 7.4), 300 to 400 mg. two hours before the experiment. The disappearance of SN 5228 from plasma was rapid so that it was necessary to administer a constant intravenous infusion of 3 to 4 μg. per min. in order to maintain the plasma level at a constant value.

Effect of urine pH

In studies designed to examine the effects of varying urine pH, a priming injection of creatinine was administered, followed by a sustaining infusion containing creatinine and sodium chloride (350 to 450 μM per min.) during an equilibration period of 30 to 60 minutes. Sodium sulfate, 100 to 200 μM per min., was included in the equilibrating infusion in many of the experiments in order to insure the excretion of acid urine. At the end of the equilibration period, collections of urine were started and the sodium chloride in the infusion was replaced by an equivalent amount of sodium bicarbonate. This generally resulted in a gradual, progressive increase in urine pH to a value between 7.5 and 8.0.

Water diuresis experiments

In experiments at acid pH, diuresis was induced by the administration of approximately 8.0 ml. per min. of a 3 to 5 per cent glucose solution delivering 100 to 200 μM per min. of sodium sulfate. In studies of urine flow at urine pH's above 6.5, the sustaining infusion contained small amounts of $NaHCO_3$, Na_2HPO_4 or K_2HPO_4.

Osmotic diuresis experiments

Osmotic diuresis was produced by the administration of increasing amounts of either 25 per cent mannitol or hypertonic sodium sulfate. One hundred μM per min. of sodium sulfate were infused in the mannitol experiments in order to fix the urine pH in the acid range. All infusions were administered intravenously at a constant rate using a Bowman infusion pump.

Urine was collected from an indwelling catheter. Collection periods were terminated by washing the bladder twice with distilled water. Urine for pH was collected anaerobically at the mid-point of each period in a silicone-coated syringe, the dead space of which was filled with mineral oil. Blood samples were collected at the mid-point of each period from an indwelling arterial needle. Clotting was prevented by the addition of heparin.

Creatinine, for the measurement of the rate of glomerular filtration, was determined in trichloroacetic acid filtrates of plasma and in diluted urine to which trichloroacetic acid was added, by a modification of the method of Folin (15). The pH of urine was measured at room temperature using a Beckman Model G pH meter and syringe-type glass electrode. No correction for temperature was applied. Ammonia in the urine was determined either by the aeration method of Van Slyke and Cullen (16) using an electrometric titration or by the microdiffusion technique of Conway (17). Atabrine® in blood and urine was measured fluorometrically by the double extraction method of Brodie, Udenfriend, Dill, and Downing (18) using heptane as the solvent for the first extraction. SN 8439 and SN 5228 were determined by the same method modified in that the fluorescence was measured in .033 N NaOH to obtain maximum sensitivity for SN 8439 and in .1 N HCl for SN 5228. Quinine was determined by a similar procedure using 1.5 per cent isoamyl alcohol in heptane for the first extraction and measuring the fluorescence in 0.1 N H_2SO_4. The specificity of these procedures for urine was examined and the material isolated from urine by these procedures found to be homogeneous, and identical with that administered, by paper chromatography in a butanol-HCl system for SN 8439 and by a 25-plate countercurrent distribution for quinine.

Plasma concentrations of the acridine derivatives, Atabrine® and SN 8439 showed no consistent trend during the course of experiments. This was to be expected since the amount metabolized or excreted is negligible with respect to the total amount in the body and the half time for disappearance from the plasma is of the order of one week. For this reason determinations of the concentrations of these acridines in the plasma were omitted in many of the experiments and only rates of excretion are presented. Quinine and SN 5228, on the other hand, disappear from the plasma more rapidly and correction has been made for the slowly declining plasma concentrations in the case of quinine by calculation of its clearance and in the case of SN 5228 by infusing it at a constant rate, under which conditions it was found that the plasma concentration remained constant.

THEORETICAL ASPECTS

The behavior of weak electrolytes in living systems is dependent upon the fact that these substances exist in solution as mixtures of unionized and ionized forms in ratios governed by the pH of the medium and the pKa of the conjugate pair. There is ample evidence that the unionized forms penetrate cell membranes relatively freely while the ions do not. This has been demonstrated for NH_3, H_2S, and cresyl blue by Harvey (19), Osterhout (20), and Irwin (21), respectively. Presumably the capacity to penetrate cell membranes is related to lipid solubility and, in general, this property is considerably reduced by ionization. These considerations led Jacobs (22) to the formulation of a general description of the mode of accumulation of weak electrolytes by cells. He concluded that the distribution of weak acids and bases between cells and surrounding fluid at equilibrium is dependent upon the pH of the medium, the pKa of the weak electrolyte and the

[1] Synthesized for us by Dr. Melvin Fish, Laboratory of Chemistry of Natural Products, National Heart Institute.

permeability of the membrane to the uncharged (lipid-soluble) species.

The concept of greater cell permeability to the unionized member of weak electrolyte conjugate pairs has been applied by a number of investigators to an analysis of the excretion of such substances in the urine with the aim of explaining the observed variation of excretion with urine pH (9–14, 23). The uncharged free base has been considered to diffuse into the tubule lumen where it is trapped by combination with hydrogen ion to form the non-diffusible ion.

The ionization of weak bases with only one ionizable group such as NH_3 (pKa 9.3) may be expressed as $B + H^+ \rightleftharpoons BH^+$ and the ionization equilibrium in the form of the Henderson-Hasselbalch equation $pH = pKa + \log \frac{(B)}{(BH^+)}$. The acridine derivatives, Atabrine®, SN 8439 and SN 5228 have two basic amino groups. The ionization is $B.B. + H^+ \rightleftharpoons H^+B.B. + H^+ \rightleftharpoons H^+B.BH^+$. The pKa of the first of these ionizations is approximately 11, so that the reaction of greatest concern in the physiologic range of pH is that involving the singly and doubly charged species. However, the singly charged acridine bases have a high degree of organic solubility and may be presumed to penetrate cells relatively easily. For purposes of brevity these also will be referred to henceforth as "free base." Quinine, although it has two ionizable groups, exists as the uncharged and singly charged species at physiologic pH since the first pKa is 8.3 whereas the second is approximately 3.0.

In a system consisting of two phases, separated by a semi-permeable membrane, and in which the pH of each phase is regulated independently, and assuming movement to occur only by diffusion, the following relationships may be considered to hold as approximations:[2]

$$M_{1,2} = K_B(B)_1 + K_{BH^+}(BH^+)_1 \quad (1)$$

and

$$M_{2,1} = K_B(B)_2 + K_{BH^+}(BH^+)_2 \quad (2)$$

where $M_{1,2}$ represents the unidirectional rate of movement or flux of free base, B, plus ion, BH^+, from phase 1 to phase 2 and $M_{2,1}$ the flux in the opposite direction; $(B)_1$ and $(B)_2$ the concentrations of the free base in phases 1 and 2, respectively; and $(BH^+)_1$ and $(BH^+)_2$ the corresponding concentrations of the ion formed by association of the base with hydrogen ion. The constants K_B and K_{BH^+} are permeability factors which include the actual permeability, the thickness of the membrane and the area available for diffusion.

At equilibrium,[3] the rate of movement in both directions is equal so that

$$M_{1,2} = M_{2,1} \quad (3)$$

and

$$K_B(B)_1 + K_{BH^+}(BH^+)_1 = K_B(B)_2 + K_{BH^+}(BH^+)_2 \quad (4)$$

[2] These are approximations in that concentrations are used instead of electrochemical potentials and solvent flow is disregarded.

[3] This will be an equilibrium only with respect to the weak electrolyte since a continuous addition of hydrogen ion will be required to maintain a pH gradient in the face of the neutralizing effect of the diffusible base.

The relative concentrations of B and BH^+ in each phase are a function of the pH of that phase and the pKa of the base.

If the membrane is completely impermeable to BH^+, ($K_{BH^+} = 0$), at equilibrium

$$(B)_1 = (B)_2 \quad (5)$$

If pH_1 and total B in phase 1 remain constant, $\log (BH^+)_2$ will be a linear function of pH_2 with a negative slope of unity. At values of pH_2 below pKa, where $(B)_2$ makes only a small contribution to the total $(B)_2 + (BH^+)_2$, the log of the sum of the total concentrations will also vary approximately as a linear function of pH with a negative slope of unity. Thus there will be an approximately tenfold increase in the concentration of weak electrolyte $(B + BH^+)$ in phase 2 for every unit decrease in pH_2.

If there is some permeability to the ion, the relationship will be non-linear and with a negative slope of less than unity. The terms containing (BH^+) do not drop out of equation 4 and the simplified form, equation 5, does not apply. Instead equation 4 may be rearranged so that

$$\frac{K_B}{K_{BH^+}} = \frac{(BH^+)_2 - (BH^+)_1}{(B)_1 - (B)_2} \quad (6)$$

This indicates that the disparity in the concentration in the two phases will be a function of the relative permeability of the membrane to the two species, as well as of pH.

Under these conditions, if $(B)_1$ and pH_1 remain constant, total B, $\Sigma(B + BH^+)$ in phase 2 will be a function of pH so long as K_B is not equal to K_{BH^+}. However, the relationship between $\log [(B) + (BH^+)]$ and pH will be complex, the slope depending upon the magnitude of $(pKa - pH_2)$. The larger the latter value, and thus the greater the excess of $(BH^+)_2$ over $(B)_2$, the flatter the relationship between $(B)_2 + (BH^+)_2$ and pH_2, since under these conditions the movement of the ion will make an appreciable contribution to the total flux even though K_B may be several orders of magnitude greater than K_{BH^+}. The negative slope of the relationship between $\log [(B)_2 + (BH^+)_2]$ and pH will approach unity at that pH at which the contribution of the ion to total movement is negligible. This will obtain at pH's close to the pKa of the electrolyte.

The influence of electrical potential across the membrane on movement of the ion has not been included in this formulation. This is justified as a reasonable approximation in the case of ammonia and quinine since movement of the uncharged free base, which is unaffected by the electrical potential gradient, presumably accounts for all but a minute fraction of the total flux. This need not be the case with the 9-amino acridines for which the pertinent equilibrium involves singly and doubly charged ions in the physiologic pH range, since movement of both ions would be modified by an electrical potential.

This general treatment of the distribution of weak electrolytes will serve as a basis for the consideration of the mode of excretion of ammonia, the acridine bases, and other weak electrolytes not transported by specific enzyme systems.[4] If the accumulation of weak bases in the urine

[4] The above derivation also may be applied to weak acids, such as salicylic, which will accumulate in alkaline solutions since the uncharged acid is lipid soluble.

TABLE I

The effect of $NaHCO_3$ on weak base excretion—dog Sp.

Time	Creatinine clearance	Urine flow	Urine pH	Rates of excretion		Clearance of quinine
				Ammonia	SN 8439	
min.	*ml./min.*	*ml./min.*		*μEq./min.*	*μg./min.*	*ml./min.*
0	Priming infusion; 2 gm. creatinine in 20 ml. H_2O					
1	Infuse 200 μM/min. Na_2SO_4. 20 mg./min. creatinine in 4.2 ml./min. .86% NaCl					
42	Change infusion to 20 mg./min. creatinine. 380 μM/min. $NaHCO_3$. 250 μM/min. NaCl in 4.2 ml./min. H_2O					
42–60	63	0.4	4.92	34.0	12.5	41.3
60–80	61	0.9	5.00	32.0	12.2	36.8
80–100	64	1.5	5.39	27.0	10.6	32.1
100–114	61	1.6	5.73	15.0	4.8	17.0
114–128	69	1.7	6.32	11.0	5.5	12.5
128–144	66	1.9	6.82	7.3	4.6	10.9
144–158	71	2.2	7.40	4.9	3.9	8.9
158–171	71	2.4	7.60	3.8	2.8	4.6
171–184	73	2.5	7.78	2.6	1.8	4.0

is governed by these equilibrium considerations, it would be anticipated that at the site at which the accumulation occurs, the concentration of weak base in the urine will be a function of pH. To the extent that no further changes in pH, volume, and weak base content of the urine occur, the concentration in the final urine will be a function of the pH. If changes in volume do take place after pH and weak base content have been established, the relationship between pH and weak base concentration will be distorted. However, if the volume flow past the last point of equilibrium remains relatively unchanged, despite alterations in volume in a more distal segment, the rate of excretion of the weak base, rather than the concentration, will be a function of pH. It will be shown that ammonia and the organic bases may be considered to move primarily as the lipid soluble or uncharged form, that equilibrium considerations apply to the excretion of these substances only in alkaline urines, and that rate of production in the case of ammonia or rate of diffusion in the case of the organic bases limits the accumulation and excretion of these substances in the acid range.

RESULTS

Effect of urine pH on base excretion

Data from experiments in which the urine pH was varied over a wide range by the infusion of

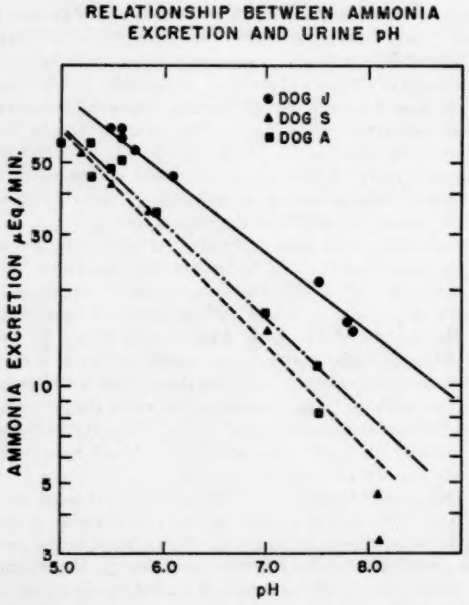

FIG. 1. THE EFFECT OF THE INFUSION OF $NaHCO_3$ ON URINE pH AND THE EXCRETION OF AMMONIA IN ACIDOTIC DOGS

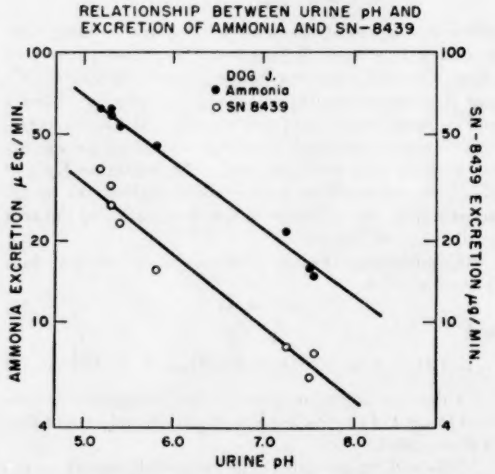

FIG. 2. THE EFFECT OF THE INFUSION OF $NaHCO_3$ ON URINE pH AND THE EXCRETION OF AMMONIA AND SN 8439

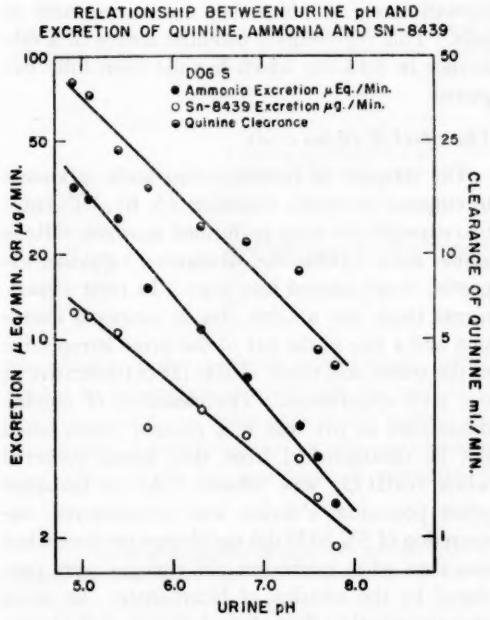

FIG. 3. THE EFFECT OF THE INFUSION OF $NaHCO_3$ ON URINE pH AND THE EXCRETION OF AMMONIA, SN 8439 AND THE CLEARANCE OF QUININE

$NaHCO_3$ are presented in Table I and Figures 1 to 5. These are representative of 27 such experiments in which the excretion of SN 8439, SN 5228, quinine, and Atabrine® either singly or in various combinations[5] was compared with that of ammonia. The uniform depression in ammonia excretion which accompanies alkalinization of the urine is illustrated in Figures 1 (ammonia only) and 2 (8439 and ammonia) and Table I. The change in the excretion of SN 8439 parallels that of ammonia as does the change in the clearance of quinine (Figure 3). It is evident that under the conditions of these experiments the slopes of lines relating the log of the excretion of ammonia and SN 8439 and the log of the clearance of quinine to urine pH are similar though not necessarily identical.[6] The slopes of these lines were independent of the absolute level of the rates of excretion in an individual dog and did not vary appreciably between experiments. As indicated by the data in Figures 4 and 5, the excretion of Atabrine® and of SN 5228 falls off sharply at pH's above 6.5 and 7.0. This was not associated with a fall in plasma concentration of these substances. It should be noted that the pKa of these compounds (± 7.4) are the ones which fall within the pH range of the urine.

Alkalinization of the urine by the infusion of potassium chloride produced results with respect to ammonia and quinine excretion and urine pH indistinguishable from those induced by $NaHCO_3$ (Table II). However, the excretion of SN 8439 diminished to a much lesser extent as urine pH rose, in some experiments actually showing an increase. Similar results with respect to ammonia were obtained when acetazoleamide was injected. The decrease in the excretion of SN 8439 under these conditions seemed uniformly to be smaller than that of ammonia in the same experiments and that of SN 8439 in other experiments in which bicarbonate was administered, but the difference was far less striking than in the experiments in-

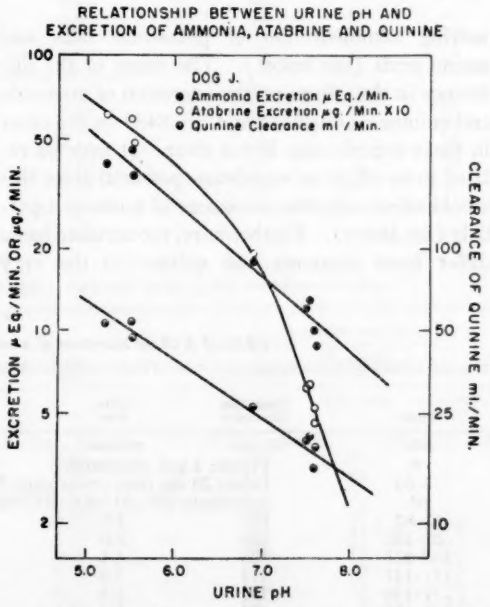

FIG. 4. THE EFFECT OF THE INFUSION OF $NaHCO_3$ ON URINE pH AND THE EXCRETION OF AMMONIA, ATABRINE® AND THE CLEARANCE OF QUININE

[5] Atabrine®, SN 8439, and SN 5228 were not administered in the same experiment since they are not easily distinguishable by the methods used.

[6] Except in the instance where the logarithm of rate of excretion is related to pH by a straight line with a negative slope of unity, there is no theoretical basis for assuming a linear relationship. Lines have been fitted to the data for empirical convenience.

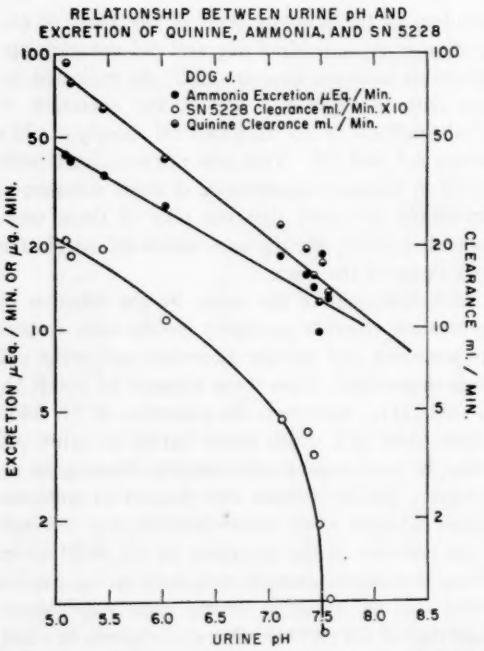

FIG. 5. THE EFFECT OF THE INFUSION OF $NaHCO_3$ ON URINE pH AND THE EXCRETION OF AMMONIA, SN 5228 AND THE CLEARANCE OF QUININE

volving administration of potassium salts and amino acids (see below). The cause of the difference in the effects on the excretion of ammonia and quinine on one hand and SN 8439 on the other in these experiments is not clear. It may be related to an effect on membrane potential since this would affect only the movement of a charged particle (see above). Furthermore, the acridine bases differ from ammonia and quinine in the very marked extent to which they are concentrated in cells. This represents a potential source of a difference in behavior which has not been fully explored.

The effect of amino acids

The infusion of certain amino acids is known to enhance ammonia excretion (5, 6). Twenty-two experiments were performed in which various amino acids (glutamine, dl-alanine, l-glycine, aspartic) were infused into dogs. In most experiments there was a brisk rise in ammonia excretion and a rise in the pH of the urine irrespective of the amino acid used. Table III is illustrative of one such experiment. The clearance of quinine diminished as pH rose in a manner which could not be distinguished from that which occurred when $NaHCO_3$ was infused. As in instances when potassium chloride was administered, the excretion of SN 8439 did not change or diminished less than when equivalent pH changes were produced by the infusion of bicarbonate. In seven experiments the effect of the infusion of dl-alanine on the excretions of ammonia, titratable acid, and bicarbonate was studied. In all, significant alkalinization of the urine occurred. Net effective acidification of the urine, as indicated by the sum of the excretions of ammonia and titratable acid minus that of bicarbonate, did not change consistently. The experiments in Tables IV and V are those in which the largest and most consistent changes occurred.

The effect of urine flow

If ammonia enters the urine by a diffusion process and equilibrium is achieved, changes in

TABLE II

Effect of KCl on excretion of ammonia, SN 8439 and quinine—dog S

Time	Creatinine clearance	Urine flow	Urine pH	Rates of excretion		Clearance of quinine
				Ammonia	SN 8439	
min.	*ml./min.*	*ml./min.*		*μEq./min.*	*μg./min.*	*ml./min.*
0	Prime: 2 gm. creatinine					
2–63	Infuse 20 mg./min. creatinine; 500 μM/min. NaCl; 100 μM/min. Na_2SO_4. 2.2 ml./min. H_2O					
63	Substitute 500 μM/min. KCl for NaCl in above infusion					
63–82	103	1.9	5.3	49.5	28.0	33.2
82–105	103	3.0	5.2	43.5	28.9	26.4
105–127	104	4.4	5.9	33.1	29.0	26.9
127–137	113	5.6	6.6	30.2	30.4	27.6
137–146	111	4.4	7.0	25.0	34.5	21.9
146–155	108	4.2	7.2	24.4	38.4	18.4
155–170	102	3.6	7.1	17.6	39.6	16.4
170–184	104	2.5	7.5	10.2	36.8	8.7

TABLE III

Effect of dl-alanine on excretion of ammonia, SN 8439 and quinine—dog J

Time	Creatinine clearance	Urine flow	Urine pH	Rates of excretion: Ammonia	Rates of excretion: SN 8439	Clearance of quinine
min.	*ml./min.*	*ml./min.*		*μEq./min.*	*μg./min.*	*ml./min.*
0	Priming injection of 2 gm. creatinine					
1	Infuse 20 mg./min. creatinine.		4.1 ml./min. .85% NaCl			
66–86	90	3.4	5.32	48	25	—
86–108	94	3.7	5.30	52	26	43
108–130	93	2.9	5.50	48	27	50
132	Add 185 mg./min. dl-alanine to above infusion					
179–197	93	4.1	6.66	96	26	26
197–219	87	4.5	7.06	115	25	21
219–234	88	6.1	6.73	124	26	18

the volume of the receiving fluid (tubule urine) should be reflected in variations in the amount of ammonia which can accumulate in the urine. Alternatively, if equilibrium is not achieved because the extent of the accumulation of ammonia is limited either by the rate at which it can diffuse out of cells or be produced within the cell, changes in urine volume will not influence the rate of excretion. The following experiments were performed to investigate these alternatives.

TABLE IV

Effect of dl-alanine on urine pH and excretion of ammonia, titratable acidity, and bicarbonate—dog J *

Time	Inulin clearance	Urine flow	Urine pH	Rates of excretion: Ammonia	Rates of excretion: Titratable acidity	Rates of excretion: HCO_3^-
min.	*ml./min.*	*ml./min.*		*μEq./min.*	*μEq./min.*	*μEq./min.*
0	Prime: 500 mg. inulin					
1	Infuse 10 mg./min. inulin, 100 μM/min. Na_2SO_4.		3% dextrose in H_2O at 7.3 ml./min.			
53–66	100	6.6	4.97	41	14	—
66–77	99	8.6	5.03	38	17	—
77–93	100	10.5	5.53	33	21	3
93–109	102	10.4	4.89	35	27	1
111	Add 300 mg./min. dl-alanine to above infusion					
109–128	105	9.6	5.49	48	27	2
128–142	111	11.1	6.49	77	17	23
142–157	105	11.8	6.56	91	20	34
157–172	101	11.5	6.56	98	22	32
172–186	97	9.2	6.73	95	26	33
186–200	81	7.5	6.81	94	22	33

* Initial plasma total CO_2 23 mM per L.

TABLE V

Effect of dl-alanine on urine pH and excretion of ammonia, titratable acidity, and bicarbonate—dog J *

Time	Creatinine clearance	Urine flow	Urine pH	Rates of excretion: Ammonia	Rates of excretion: Titratable acidity	Rates of excretion: HCO_3
min.	*ml./min.*	*ml./min.*		*μEq./min.*	*μEq./min.*	*μEq./min.*
0	Prime: 2 gm. creatinine					
1	Infuse 20 mg./min. creatinine, 100 μM/min. Na_2SO_4.		145 μM/min. NaCl.		11.5 ml./min. H_2O	
60–76	97	3.9	4.91	116	85	—
76–86	96	10.1	4.87	115	83	—
89	Add 200 mg./min. dl-alanine to above infusion					
86–100	98	11.8	5.02	128	62	—
100–111	106	11.3	5.79	171	21	4
111–121	109	11.1	6.30	178	14	12
121–132	105	12.1	6.45	174	16	18
132–143	107	13.6	6.53	174	12	34
143–154	91	14.3	6.60	181	9	43

* Initial plasma total CO_2 10 mM per L.

TABLE VI

Effect of Na_2SO_4 diuresis—dog A

Time	Creatinine clearance	Urine flow	Urine pH	Rates of excretion	
				Ammonia	SN 8439
min.	*ml./min.*	*ml./min.*		*μEq./min.*	*μg./min.*
0	Prime: 2 gm. creatinine				
	Infusion I: Right leg—25 mg./min. creatinine; 100 μM/min. Na_2SO_4, H_2O 1 ml./min.				
	Infusion II: Left leg—300 μM/min. Na_2SO_4 in H_2O, 2 ml./min.				
40–55	73	4.3	5.19	38	18
55–65	74	6.0	5.28	38	19
65–77	71	6.4	5.48	39	18
83	Increase speed of Infusion II to 4 ml./min.				
99–112	73	8.0	5.26	40	16
112–124	73	8.7	5.20	41	17
127	Increase speed of Infusion II to 6 ml./min.				
124–131	—	9.5	5.02	42	17
131–145	—	10.4	5.32	—	20
146	Increase speed of Infusion II to 10 ml./min.				
145–157	—	12.1	5.22	39	18

TABLE VII

Effect of mannitol diuresis—dog H

Time	Creatinine clearance	Urine flow	Urine pH	Rates of excretion	
				Ammonia	SN 8439
min.	*ml./min.*	*ml./min.*		*μEq./min.*	*μg./min.*
0	Prime: 2 gm. creatinine				
1	Infuse: 100 μM/min. Na_2SO_4; 20 mg./min. creatinine; 1.0 ml./min. H_2O, left leg				
10	Infuse: 25% Mannitol at 2.5 ml./min., right leg				
29–53	68	8.1	4.72	32	21
53–64	65	8.7	4.68	32	21
66	Increase speed of Mannitol infusion to 5 ml./min.				
64–76	62	9.3	4.68	31	20
76–85	79	13.3	4.68	28	25
85	Increase speed of Mannitol infusion to 8.0 ml./min.				
85–95	71	13.1	4.68	25	23
95–104	69	14.9	4.68	27	22

Irrespective of the means of inducing diuresis, variations in urine flow exerted no influence on the rates of excretion of ammonia or other organic bases if the urine were maintained acid throughout. This is illustrated for water diuresis in Figure 6. Data for the excretion and concentration of SN 8439 were similar. Tables VI and VII are illustrative of experiments in which osmotic diuresis in the acid range did not alter base excretion.

As noted earlier, experiments of a similar nature were difficult to perform successfully when the urine pH was higher. However, urine flow does modify the rates of excretion of the weak bases in the alkaline range. With respect to water diuresis, this conclusion was reached by comparing excretion rates at the same pH but different urine flows. In the experiments summarized in Table VIII the periods were not necessarily consecutive. Figure 7 is constructed from data obtained during

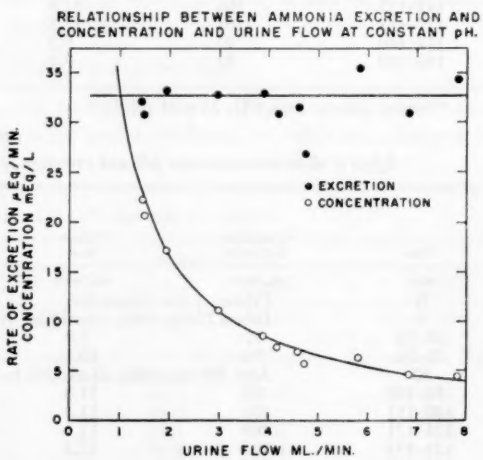

FIG. 6. THE EFFECT OF H_2O DIURESIS ON THE EXCRETION AND CONCENTRATION OF AMMONIA

Urine pH varied from 5.1 to 5.3.

osmotic diuresis produced by the infusion of 25 per cent mannitol. Both ammonia excretion and quinine clearance rose as urine flow increased despite relative constancy of pH between 6.9 and 7.2. It is difficult to specify the exact acidity at which a transition occurs from the range in which changes in urine flow do not modify excretion to that in which urine flow is a factor. However, rate of flow seemed to influence weak base excretion when the urine pH rose above 6.0 to 6.25. It may be noted (Table VIII) that in three of the four experiments with urine pH's below 6.25 the excretion of ammonia changed with urine flow whereas that of SN 8439 did not.

TABLE VIII

Effect of urine flow in water diuresis at high urine pH

Experiment	Urine pH	Urine flow	Excretion of ammonia	Excretion of SN 8439	Clearance of quinine
		ml./min.	*μEq./min.*	*μg./min.*	*ml./min.*
1*	7.93	1.1	2.3	7	
	7.84	2.2	3.9	11	
2	7.92	8.4	2.3	1.6	9.9
	8.00	3.0	1.8	1.0	4.6
3	7.72	4.9	6.4	6.5	5.0
	7.76	2.2	2.5	3.6	2.1
4	7.51	13.3	6.1		6.0
	7.54	1.2	2.3		1.1
5*	7.33	4.1	2.5	4.0	
	7.35	8.0	5.0	5.7	
	7.38	3.7	2.4	3.8	
6	7.06	13.7	11		10
	7.09	3.1	7.5		4.0
7	6.95	4.0	11	21	
	6.82	1.1	5.2	19	
8*	6.88	1.7	7.1	11	
	6.82	3.4	11	19	
9*	6.41	2.3	13	12	
	6.28	6.5	21	18	
10	6.25	1.2	8.8	13	
	6.27	3.7	20	13	
11*	6.10	1.9	15	11	
	6.13	5.3	20	12	
12	6.08	9.1	26	19	
	6.09	4.3	19	13	
13*	6.06	6.2	25	31	
	6.11	10.1	32	33	

* Data from these experiments represent consecutive periods.

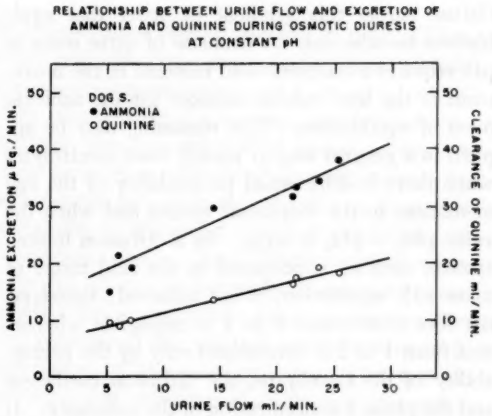

FIG. 7. THE EFFECT OF MANNITOL DIURESIS ON THE EXCRETION OF AMMONIA AND THE CLEARANCE OF QUININE
Urine pH varied from 6.9 to 7.2.

DISCUSSION

The reproducible relationship between the excretion of the weakly basic electrolytes and urine pH and the similarity of behavior of bases with similar ionization constants are consonant with the view that these substances diffuse out of cells primarily in the lipid soluble form and accumulate in the urine as the ion to which the cell membrane is less permeable. This mechanism is operative throughout the entire range of urine pH, though important differences exist in the mode of accumulation in the acid and alkaline ranges of urine hydrogen ion concentration.

The excretion of weak bases in acid urines

In any system in which the time available for diffusion is limited by the movement of one phase past the other, as in the renal tubule, equilibrium may not be achieved when this requires movement of a large amount of substance across a small concentration gradient. This is the situation with regard to ammonia. The concentration gradient over which all the ammonia must diffuse is the difference between the concentration of free ammonia in the cell and free ammonia in the urine. Each of these is only a very small fraction of the total ammonia in the respective phases. Urine pH exerts a minimal influence on the concentration gradient for free ammonia but has a considerable influence on the amount of ammonia which must

diffuse across this gradient in order that equilibrium be achieved. A decrease of three units in pH requires a thousand fold increase in the movement of the lipid soluble member for the achievement of equilibrium. This reasoning may be applied in a general way to weakly basic electrolytes when there is differential permeability of the cell membrane to the individual species and when the value $pH_1 - pH_2$ is large. In a diffusion limited system, such as is proposed in the acid range of urine pH, equilibrium is not achieved; therefore, the flux from phase 2 to 1 is negligible whereas that from 1 to 2 is determined only by the permeability of the membrane, the diffusion coefficient and the phase 1 concentration of the substance. It thus makes little difference what the characteristics of phase 2 may be as to either volume or pH so long as the volume is large enough and the pH low enough. In the case of a substance, such as ammonia, which is produced in phase 1 (the cell) the capacity to produce may not be adequate to replace it as rapidly as it leaves. The concentration in phase 1 will fall and it will enter phase 2 at a rate equal to the rate at which it can be produced. Such a system might be called production-limited and again the rate of entry into phase 2 will not be affected by changes in phase 2.

Experiments designed to determine whether such considerations apply in the dog [7] have shown that in the acid range of urine pH changes in volume of urine do not have an effect on the excretion of weak electrolytes. This is the basis for the conclusion that equilibrium is not achieved in this pH range. The limiting factor in the case of ammonia is presumably the rate of intracellular production, in the case of the organic bases rate of diffusion. For either type of substance (ammonia or exogenous base) the contribution of each individual segment of the tubule urine is maximal so long as the urine pH is low enough. The urinary pH below which production limits the excretion of ammonia would not likely be identical with that at which the entry of the various exogenous bases becomes diffusion-limited. However, although the data do not define the various pH ranges sufficiently to make possible a precise separation, it would appear from Table VIII that a difference between ammonia and SN 8439 excretion probably exists in the range of urine pH between 6.0 and 6.25. It is also unlikely that the curve relating urine pH to base excretion should have the same slope throughout the entire pH range in view of the differences in mechanism of accumulation at varying pH's (see below). The data have sufficient scatter to mask considerable differences in slope at the extremes of pH.

In order to account for the observed effect of alterations of pH on ammonia excretion in acid urines in contrast to the lack of effect of volume changes, it is necessary to consider that diffusion of ammonia out of cells is not limited to an isolated segment of the tubule but extends over an area various portions of which may differ in their contribution depending on the luminal pH. The longer the segment of tubule over which the urine pH is maintained in the production limited range the greater the number of cells which can contribute maximally to ammonia excretion. Urine excreted at a particular acid pH must have had hydrogen ions added more rapidly or have had a lower initial concentration of buffer and have been acid over a longer segment of tubule than any less acid urine. If the urine of lower pH has been sufficiently acid to receive ammonia maximally (*i.e.*, was in the pH range where production limits ammonia entry) over a longer segment of tubule it will accumulate more ammonia.

The excretion of weak bases in neutral and alkaline urines

In more alkaline urines, alterations in urine volume exert an appreciable effect on ammonia excretion. Consequently, equilibrium considerations presumably apply at the site of entry of weak bases in the urine. However, even in this range the data indicate that rate of excretion is more closely related to pH than is concentration. This has been mentioned previously (see above). Such a finding is in accord with the view that final volume adjustments in the kidney tubule occur at a site distal to electrolyte transport (25, 26) and that variations in ultimate urine volume are greater than those at the site of entry of the weak bases studied. At some point in the tubule, urine pH

[7] The applicability of these data to man is clear since experiments relating urine pH to ammonia and Atabrine® excretion have given similar results, as have experiments in which urine flow was modified. Quinine clearance, on the other hand, was not related to urine pH as it is in the dog (24).

may be considered to determine the concentration of ammonia; more distally, in an electrolyte impermeable area, abstraction of water distorts the relationship between electrolyte concentration and pH but not that between rate of excretion and pH.[8]

If diffusion equilibrium of weak bases is achieved, the negative slope of the line relating total excretion to pH should approach unity as an approximation when the ionized form is completely non-permeating. The slope of less than unity observed with ammonia, quinine and SN 8439 may therefore be consequent to slight but significant movement of the ionized form. On the other hand, the urinary excretion of Atabrine® and SN 5228, both of which have pKa's in the neighborhood of 7.4, diminishes much more rapidly at high urine pH's than that of the other compounds studied (Figures 4 and 5). This would be expected even if there were some permeation by the ion since at pH's close to the pKa the relative contribution of the less diffusible form to the total movement is negligible because of the limited concentration at which the ion is present. Under these circumstances the negative slope of the line relating log of excretion to pH should approach unity. This condition is never achieved in the case of SN 8439 or ammonia since their pKa's exceed the maximal urine pH attainable.

The general formulation of the equilibrium distribution of weak electrolytes was based on the assumption that the concentration of ammonia in the cell is constant despite continuous diffusion into the urine. The applicability of this assumption is supported by the parallelism between the excretion of SN 8439, quinine and ammonia, since the interstitial concentrations of the exogenous bases are virtually unaffected by changes in urinary excretion. This implies that the net production of ammonia is more or less equal to the rate of excretion. If the net production of ammonia were not determined by its rate of loss from cells, excretion would be unaffected by urine pH except for transient periods of accumulation in cells and unloading accompanying rises and falls in urine pH, respectively.

The maintenance of a constant intracellular concentration requires either that formation of ammonia occur by a reversible enzymatic process or that ammonia formed in the course of an irreversible reaction be channeled into both the urine and a metabolic pool. Intracellular concentration would not change appreciably if ammonia were produced by a reversible reaction since under these conditions end product concentration is a determinant of the reaction rate. The second possibility that diffusion of NH_3 into the urine (and blood) and cellular utilization are, in a sense, competitive processes is not unreasonable. If such a mechanism were involved, when less NH_3 entered the urine, as at high pH, an increase in the rate of utilization by the second process would tend to stabilize the intracellular concentration. In fact, the alternative, the production of ammonia by a reversible reaction, constantly at or near equilibrium, is not consonant with the role of enzyme adaptation in increasing ammonia excretion at all urine pH's (1–3). At the equilibrium of a reversible process the ammonia concentration in cells would be unaffected by changes in enzyme concentration since enzymes speed the attainment of equilibrium but do not affect the concentrations of the various reactants at equilibrium. Either alternative mechanism of concentration stabilization would account for ammonia excretion when the urine is sufficiently acid since the rapid loss from cells to urine would lead to production at a maximal rate and minimal utilization in alternative pathways.

The effect of the administration of amino acids is presumably an augmentation of the rate of production and an increase in the intracellular concentration of ammonia. The present amino acid experiments do not, in themselves, permit a conclusion as to the mechanism by which the ammonia enters the urine. The observed rise in pH could be attributed to the titration of hydrogen ion by the free ammonia diffusing into the urine. Alternatively, it is recognized that these changes could also be due to a direct exchange of NH_4^+ for Na^+ if the exchanging NH_4^+ were substituted for an equivalent amount of exchanging hydrogen

[8] Although, in a tubule permeable to CO_2, the urine pH will be affected by the process of concentration (27), a ten-fold diminution in volume will change the pH by as much as one unit if the urine is completely unbuffered. It is unlikely that this degree of concentration occurred in these experiments and buffer is never completely absent from the urine.

ion.[9] In any event, the fundamental physiologic role ascribed to ammonia excretion, fixed base conservation, was not evident in these experiments. For the most part, there was little difference between the increase in ammonia excretion and the change in the excretion of bicarbonate minus the change in titratable acid. Under physiologic conditions, the excretion of ammonia presumably serves to promote the reabsorption of sodium in exchange for hydrogen by minimizing the pH gradient between cells and urine (9). The reaction of NH_3 and H^+ to form NH_4^+ in the tubule urine is thought to facilitate further secretion of hydrogen ions in exchange for sodium. In such experiments as those of Lotspeich and Pitts (5) in which no change in urine pH or titratable acid accompanied the increase in ammonia excretion when amino acids were infused, such a mechanism may be considered to have been operating. The reason for the difference between the results of Lotspeich and Pitts (5) and those of the present experiments with respect to urine pH is not clear. In any case, no base conservation can result if ammonium ion merely replaces hydrogen ion in the urine. The mechanisms for hydrogen ion and ammonia secretion must be coextensive and the entry of ammonia permit the further secretion of hydrogen ion.

SUMMARY AND CONCLUSIONS

Experiments have been performed to investigate the mechanism of excretion of ammonia and other weak bases in the dog.

The excretion of these substances (ammonia, quinine, Atabrine®, SN 8439 and SN 5228) diminishes as urine pH rises when the change is effected by the infusion of $NaHCO_3$. Alkalinization of the urine by the injection of potassium chloride or acetazoleamide results in a similar diminution in ammonia and quinine excretion. The effect on the excretion of SN 8439 is less marked.

The accumulation of organic bases in the urine is apparently dependent upon the differential permeability of the tubule cell membrane to the lipid soluble and insoluble members of the ion pairs. These substances diffuse across the membrane as the free base (or singly charged member in the case of Atabrine®, SN 8439 and SN 5228) and accumulate in the urine as the less permeating lipid insoluble species. In the acid range of urine pH accumulation is limited by the maximal rate of intracellular production in the case of ammonia and by the maximal rate of diffusion out of cells in the case of the exogenous bases. Consequently, alterations in urine flow do not affect the rate of excretion. In more alkaline urines, however, urine flow influences excretion rate so that diffusion of the bases between cells and tubule urine presumably approaches equilibrium. In either instance, the relationship between urine pH and rate of excretion rather than concentration of base indicates that final abstraction of water occurs at a site distal to that at which weak electrolyte content is determined.

Amino acids augment ammonia excretion by enhancing the rate of production of ammonia and by elevating the intracellular concentration. It has not been possible to distinguish between the various mechanisms which could account for the rise in urine pH noted in these experiments. In any event, base conservation was not effected, since in general the rise in ammonia excretion did not appreciably exceed the sum of the fall in titratable acid plus the rise in bicarbonate excretion.

It is concluded that the excretion of ammonia and weak bases is not ascribable to active transport of these substances into the urine but to passive diffusion conditioned by the acidification of the urine.

[9] All agents known to inhibit hydrogen ion secretion which have been studied have been found to increase potassium excretion (28). The fact that potassium excretion did not increase when urine pH rose as a result of amino acid administration is in accord with the view that the rise in urine pH may not be attributable to inhibited acidification.

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CERTAIN ASPECTS OF TYROSINE METABOLISM IN THE YOUNG. I. THE DEVELOPMENT OF THE TYROSINE OXIDIZING SYSTEM IN HUMAN LIVER [1]

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The major pathway for tyrosine catabolism in the animal is the non-oxidative transamination of tyrosine to p-hydroxyphenylpyruvate and its subsequent oxidation to homogentisate. The homogentisate is oxidized to fumarylacetoacetate (maleylacetoacetate), which is finally hydrolyzed to fumarate (malate) and acetoacetate. The enzyme system responsible for this catabolism, tentatively called the tyrosine oxidizing system, is soluble and a partial purification can be accomplished with high speed centrifugation of homogenates of liver (1) and kidney (2). When these preparations are used it has been observed (3) that one mole of tyrosine will be completely oxidized to one mole of acetoacetate and one mole of fumarate, with the utilization of two moles of oxygen. In order to obtain maximal tyrosine transaminase activity it is necessary to add α-ketoglutarate and pyridoxal phosphate *in vitro*. The oxidation of the formed p-hydroxyphenylpyruvate requires ascorbic acid (1, 4, 5) as a cofactor or an equivalent oxidation-reduction substitute such as dichlorophenolindophenol (6, 7).

Clinically, it was shown (8, 9) that premature infants maintained on a diet high in protein without added ascorbic acid excreted p-hydroxyphenylpyruvic acid in their urine. Similarly, p-hydroxyphenylpyruvic acid was excreted in the urine of scorbutic adults (10, 11). The addition of ascorbic acid to the diet of both groups resulted in the disappearance of this substance from the urine. It was also observed that when large doses of pteroylglutamic acid were given to scorbutic guinea pigs (12), monkeys (13), and premature infants (14) there was a decrease in keto acid excretion. ACTH, after a latent period, also decreased the excretion of p-hydroxyphenylpyruvic acid by premature infants (15) and scorbutic guinea pigs (16). The mode of action of pteroylglutamic acid and ACTH is still obscure. In contradistinction to the above findings it was observed that in two of the three reported adult cases of tyrosinosis there was no effect of ascorbic acid on the urinary excretion of tyrosine or of p-hydroxyphenylpyruvic acid (3).

These investigations are concerned with the relationship of growth of the organism to the development and action of the tyrosine oxidizing system. The data obtained from premature infants, immature rats, and older subjects will be used in an effort to explain the previous results in terms of a developing enzyme system.

METHODS

1. *Subjects*

Samples of liver from premature infants, newborn infants and adults were obtained at autopsy.[2] All donors were clinically free of hepatic disease and their livers showed no gross evidence of liver pathology. The mothers of all premature infants had uneventful pregnancies. Special precautions were taken to obtain the livers from the premature infants as soon after death as possible. These infants were removed to the morgue refrigerator; the autopsy was promptly performed and liver samples obtained.

Livers from all subjects were immediately placed in a chilled beaker which was kept on ice and transported to the laboratory for immediate preparation. The data on human subjects are summarized in Table I.

Samples of adult animal livers were obtained from the Long-Evans strain of rats following sacrifice by a blow to the head. The liver was quickly removed and rinsed with ice-cold 0.9 per cent KCl to remove adherent

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[2] The authors acknowledge the assistance of Dr. Arthur Carlson of the Department of Pathology, and the Pediatric House Staff in obtaining the specimens.

TABLE I

Summary of clinical data

Subject	Sex	Age	Estimated gestational age—*days*	Wt. *kg.*	S.A. M^2	Cause of death	Autopsy hours after death
I. Premature infants							
P—10	M	4 hrs.	178	0.89	.084	Immaturity, atelectasis	1¼
P—12	M	51 hrs.	233	1.94	.151	Immaturity, atelectasis	1¼
P—14	M	168 hrs.	229	1.48	.124	Immaturity, atelectasis	7
P—15t*	M	24 hrs.	—	1.40	.119	Immaturity, atelectasis, hyaline membrane disease	3
P—21	F	55 hrs.	176	0.87	.083	Immaturity, atelectasis, multiple hemorrhages	23
P—23t	M	7 hrs.	193	1.01	.093	Immaturity, atelectasis	3½
P—24t	M	28 hrs.	193	1.27	.110	Immaturity, multiple hemorrhages, atelectasis, emphysema	7
P—31	F	6 hrs.	228	1.13	.101	Immaturity, atelectasis, hyaline membrane disease	3
P—32	F	7½ hrs.	172	0.72	.072	Immaturity, atelectasis, subdural hemorrhages	3½
P—34	F	16 hrs.	189	0.75	.074	Immaturity, kernicterus, atelectasis, ulcerations of ileum	6
P—41	M	10 hrs.	169	0.72	.072	Immaturity, atelectasis, hyaline membrane disease	2½
II. Full-term infants							
NB—19	M	36 hrs.		2.76	.198	Atelectasis with multiple visceral hemorrhages	3½
NB—35	M	20 hrs.		3.22	.221	Aspiration pneumonia, multiple hemorrhages	8
III. Child							
C—23	F	11 yrs.		28.4	.892	Multiple congenital anomalies, hydronephrosis, agenesis of left kidney, hypertensive cardiovascular disease	9½
IV. Adults							
A—13	M	55 yrs.		64.0	1.82	Astrocytoma—resected	14
A—13	F	70 yrs.		111	1.56	Diffuse hemorrhagic encephalopathy, cardiovascular disease	14
A—22	F	65 yrs.		39.0	1.46	Coronary arterial occlusion with myocardial infarction	12
A—25	M	37 yrs.		56.0	1.77	Hypertensive cardiovascular disease	5
A—29	M	58 yrs.		56.0	1.70	Fibro-caseous pulmonary tuberculosis, cor pulmonale with arteriosclerotic heart disease	10½

* t = twin.

blood prior to grinding. Infant and newborn rats were sacrificed by decapitation or decerebration and exsanguinated. Fetal rats were removed from a pregnant animal and decapitated. The livers from all of the groups were removed immediately for tissue preparation except in the case of those rats which were permitted to remain for varying periods in the cold room (temperature 2 to 4°C) or at room temperature (26 to 28°C) to assay the effect of these factors on the hepatic tyrosine oxidizing system. Only litter mates were compared in these experiments.[3]

[3] The effect of varying conditions following autopsy on the tyrosine oxidizing system was studied by killing infant rats by different techniques and exposing them after death to room temperature for varying time intervals (17). No differences in oxidative activity resulted when the animals were sacrificed with and without complete exsanguination. Rats exposed to room temperature for three hours showed no change in oxidative activity, but exposure for six hours resulted in a decrease in activity. The decrease was proportional to a rising level of homogentisic acid which was undoubtedly due to the decrease in the pH of the liver and the subsequent inhibition of homogentisic acid oxidase (3).

2. Preparation of tissue

Human and rat livers were immediately placed in a dish on ice in the cold room for prompt and complete preparation of samples. Generally, the human liver was pressed through a hand-tissue press with a sieve of 1 mm. diameter holes to remove connective tissue mechanically.

The resultant mash was ground for two minutes in a Waring Blendor with one to three volumes of ice-cold 0.9 per cent KCl. No difference in oxidative activity was observed whether the homogenates were prepared in the Waring Blendor or the Teflon-glass homogenizer. The rat tissue was homogenized in the Teflon homogenizer because of the small amount of sample. In the case of the infant rats, it was necessary to pool two to four livers to provide a sufficient quantity of tissue. Following homogenization the volume was measured and aliquots were centrifuged in the Sorvall SS-1 centrifuge at 18,000 × g for 15 minutes. The resultant supernatant fluid or soluble fraction was collected and the volume measured. It was found that a force of 100,000 × g for 30 minutes in the Spinco centrifuge Model L did not decrease the oxidative activity, indicating that the enzyme was soluble (1). In some instances the soluble fraction was dialyzed against tap water in the cold overnight and the activity of the dialyzed material was measured. This modification of the procedure suggested by Knox and LeMay-Knox (1) was found to be simpler and less erratic than the use of acetone powders (18) or sucrose as a solvent (19).

3. Measurement of oxidative activity

Measurements were made with the total homogenate, the soluble fraction resulting from high-speed centrifugation and the dialyzed soluble fraction. Samples (0.30 to 1.00 ml.) were placed in previously prepared Warburg flasks with sidearms, the flask contents being essentially those described by Knox and LeMay-Knox (1). Besides the liver samples, the flasks contained 2.0 ml. of 0.2 M phosphate buffer pH 7.4, 8 μM of tyrosine, and 0.2 ml. of an ascorbic acid solution (.250 to 5 mg.) adjusted to pH 7.4. The sidearm contained 0.3 ml. of α-ketoglutarate (30 μM) adjusted to pH 7.4 and 0.2 ml. of pyridoxal phosphate (60 μg.).[4] The center well contained 0.2 ml. of 20 per cent KOH on a strip of filter paper in order to absorb carbon dioxide. Glass distilled water was added to the main central compartment to bring the total volume to 3.7 ml. A control flask lacking tyrosine but containing all of the other reactants was run with each tissue sample. The "endogenous" oxygen uptake was measured and deducted from the oxygen uptake of the flasks containing tyrosine. Measurements were made at 37.3°C and were started after an equilibration period of 10 minutes at which time the contents of the sidearms were tipped into the main portion and the stopcocks were closed. Readings were taken at 10-minute intervals for a total period of 60 minutes. When measurements were made in an atmosphere of nitrogen the flasks were "gassed" on the bath during the 10-minute equilibration period. Following closure of the stopcocks, readings were taken to preclude the extraneous uptake of oxygen.

[4] The authors wish to thank Dr. Kenneth Tsuboi for his generous contribution of pyridoxal phosphate.

4. Chemical determinations

Nitrogen determinations were made on aliquots of the original liver tissue by the Ma-Zuazaga modification of the micro-Kjeldahl method (20). An additional aliquot of the total homogenate was treated according to a modification of the Schneider trichloroacetic acid fractionation method (21) and desoxyribonucleic acid was determined on the fraction remaining after the sample was first treated with an equal volume of ice-cold 10 per cent trichloroacetic acid and alcohol-ether. The final precipitate was heated with 5 per cent trichloroacetic acid for 15 minutes at 90°C and the desoxyribonucleic acid was determined in the supernatant fluid with the diphenylamine reaction (22).

Immediately following completion of measurements of oxidative activity, the contents of the Warburg flasks were removed and aliquots were placed in equal volumes of trichloroacetic acid (TCA) and metaphosphoric acid to make 5 per cent solutions. The protein precipitate was removed from both of these suspensions by centrifugation. From the supernatant fluid of the TCA precipitation, an aliquot was taken for the determination of Briggs reacting material (p-hydroxyphenylpyruvic acid, homogentisic acid, and also ascorbic acid) according to a modification of the method of Medes (23). In order to remove interference from ascorbic acid an aliquot from the trichloroacetic acid solution was extracted three times with peroxide-free ether. This ether extract containing p-hydroxyphenylpyruvic acid and homogentisic acid was evaporated to dryness and the Briggs' reaction was performed as outlined by Medes. Similar treatment of pure solutions of p-hydroxyphenylpyruvic acid gave recoveries of approximately 70 to 75 per cent. The original trichloroacetic acid filtrate prior to ether extraction was utilized directly for the determination of homogentisic acid by the iodometric method of Neuberger and Webster (24).

In order to determine micro-quantities of homogentisic acid, .001 N thiosulfate and .001 N iodine were used. To estimate maleylacetoacetic acid, another aliquot of the trichloroacetic acid extract was read in the Beckman DU spectrophotometer with a blank prepared from the TCA extract of the specific control flask. Acid solutions depress the ultraviolet absorption of maleylacetoacetic acid (3) but readings of samples showing oxidative activity at wave lengths of 300 mμ to 345 mμ showed a band which was not present in the tissue blanks or in samples incubated in an atmosphere of nitrogen. The absorption of the p-hydroxyphenylpyruvic acid was compensated for by the addition of an equivalent amount of p-hydroxyphenylpyruvic acid to the tissue blank against which the samples incubated in nitrogen were read. Under these conditions the presence of an absorption curve at 300 mμ to 345 mμ was taken as an estimate of the presence of maleylacetoacetic acid.

The metaphosphoric acid extract was used for measurement of p-hydroxyphenyl compounds (24): tyrosine and p-hydroxyphenylpyruvic acid. To avoid turbidity, these samples were read as soon as they became optically

constant, approximately three minutes after the sodium nitrite was added. The remainder of the metaphosphoric acid extract was used for the formation and determination of 2-4 dinitrophenylhydrazones according to the methods of Cavallini and Frontali (25). The metaphosphoric acid filtrate was first extracted three times with peroxide-free ether in order to remove p-hydroxyphenylpyruvic acid and leave the α-ketoglutaric acid in the residue. It was found that α-ketoglutaric acid, being in much greater concentration than the aromatic keto acid, interfered with the chromatograms in the absence of ether extraction. The hydrazones which were formed on addition of 2-4 dinitrophenylhydrazine were chromatographed with n-butanol (25) as the moving solvent on Whatman No. 3 strips. A few drops of formic acid were placed in the drum to prevent tailing. The p-hydroxyphenylpyruvic acid migrated as two spots due to the syn-anti configuration and gave R_f values of 0.83 and 0.96. Pure homogentisic acid and p-hydroxyphenylpyruvic acid for use as standards were obtained through the courtesy of Dr. W. Eugene Knox and the latter was also purchased from the Homberg Chemiewerk, Frankfurt am Main.

RESULTS

The data in Table I show that 10 of the 11 liver specimens from premature infants were obtained within 7 hours after death. The weight of the infants ranged from .72 kg. to 1.94 kg. and their gestational age, from 169 to 233 days with a mean of 196 days. Specimens from full-term infants and adults were obtained within 14 hours after death.

The data in Table II present the oxidative activities obtained with the total homogenate, the soluble fraction derived from high speed centrifugation and the dialyzed soluble fraction. Since this enzyme system is soluble it will be noted that when enzyme activity is rated on the basis of nitrogen it consistently increased in the adult and full-term infant livers as the nonactive particulate matter was removed. In contrast, this increase was noted in the soluble fraction of only 2 of the 11 premature infant livers and in terms of the nitrogen standard was negligible. The liver from the premature infant obtained 23 hours after death (P-21) showed virtually the highest activity both on the basis of wet weight and nitrogen. The hepatic activity of the full-term infants and the adults was 3 to 20 times greater than that of

TABLE II

The activity of the tyrosine oxidizing system in fractions of human liver

Subject	Homogenate $\mu l.\ O_2/30'/gm.\ wet\ wt.$	Homogenate	Soluble fraction $\mu l.\ O_2/30'/mg.\ N$	Dialyzed soluble fraction
I. Premature infants				
P—10	0	0	0	0
P—12	6	0	2	2
P—14	59	2	4	4
P—15	0	0	0	—
P—21	81	4	4	—
P—23	51	2	1	2
P—24	9	0	0	—
P—31	—	—	—	1
P—32	18	1	1	1
P—34	0	0	0	—
P—41	—	—	0	—
Average	25	1	1	1
II. Full-term infants				
NB—19	51	2	5	—
NB—35	63	3	5	5
Average	57	3	5	5
III. Adults				
C—23	240	9	26	17
A—13	732	26	36	27
B—13	192	8	21	—
A—22	164	7	11	—
A—25	204	7	13	25
A—29	316	10	28	57
Average	308	12	23	32

TABLE III

Activity of the tyrosine oxidizing system of the soluble fraction with various standards of reference

	Oxygen uptake									Total liver activity $\mu l.\ O_2/30'/M^2$ body S.A. $\times 10^{-3}$		
	Number of subjects	Range	Average	Number of subjects	Range	Average	Number of subjects	Range	Average	Number of subjects	Range	Average
	$\mu l./30'/gm.$ wet wt.			$\mu l./30'/mg.\ N$			$\mu l./30'/mg.\ DNA$*					
Premature infants	10	0–80	21	11	0–4	1	2	6–15†	11	10	0–45	15
Full-term infants	2	73–79	76	2	5–5	5	2	22–34	28	2	44–51	48
Adults	6	140–612	343	6	11–36	23	1		135†	6	152–483	318

* The DNA was determined on the total homogenate; as expected, no DNA was found in the soluble fraction.

† The values for $\mu l.\ O_2/30'/mg.$ DNA of 6 and 15 were obtained from premature infants P—32 and P—21, respectively, and the value of 135 $\mu l.\ O_2/30'/mg.$ DNA was obtained from the adult A—29.

the premature infants and could be considered infinitely higher when only negligible activity was present in the liver of the premature infant. These data indicate the complete absence or only slight presence of tyrosine oxidation in livers of premature infants.

Table III shows the mean values for oxidative activity, based on liver wet weight, nitrogen, desoxyribonucleic acid and total liver activity per square meter of body surface. The data show that irrespective of the standard of reference used for oxidative activity, the livers from premature infants practically always showed minimal activity and they in no instance even approximated the activity of the adult or full-term infant livers.

In Figure 1 the comparative effects of 500 $\mu g.$ of ascorbic acid on the oxidation of tyrosine by the soluble fraction of the liver of the premature infant and the adult are shown. It is clear that this concentration maximally stimulated the adult liver

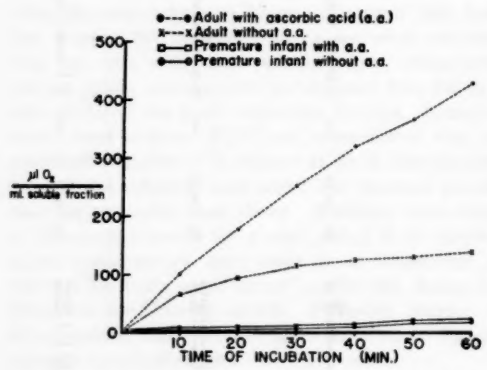

FIG. 1. THE EFFECT OF ASCORBIC ACID ON THE ACTIVITY OF THE TYROSINE OXIDIZING SYSTEM OF HUMAN LIVER

but showed no effect on the activity of the soluble fraction of the liver from the premature infant. Similar results were obtained with concentrations up to 5 mg. of ascorbic acid.

Table IV shows the effect of adding the supernatant fluid of boiled adult rat liver homogenate and the filtrate from the ultrafiltered rat liver soluble fraction to the soluble fraction of the liver of the premature infant. Neither of these additions resulted in an increase in the oxidative activity of the liver of the premature infant. The addition of the soluble fraction of the liver of the premature infant had no significant effect on the oxidative activity of the adult rat liver indicating no inhibitory activity.

Table V represents values for the various intermediate compounds which resulted from the oxidation of tyrosine by the soluble fraction of premature infant liver and adult liver. The adult liver selected, and used only in these observations, was obtained from an individual with carcinoma of the kidney and widespread metastases. As a consequence of the pathological changes in the liver there was a decreased oxidative activity, 28 $\mu l.\ O_2/30'/ml.$ (84 $\mu l.\ O_2/30'/gm.$ wet wt.) compared to the previously noted average of 102 $\mu l.\ O_2/30'/ml.$ (308 $\mu l.\ O_2/30'/gm.$ wet wt.) for normal adult liver (Table II). This drastic reduction in oxidative activity in the pathologic adult liver nevertheless resulted in a value which remained considerably elevated above that of premature infant livers (84 $\mu l.\ O_2/30'/gm.$ wet wt. compared to the previously noted average value of 25 $\mu l.\ O_2/30'/gm.$ wet wt. for premature infant liver—Table II). A pathologic adult specimen was used for the analysis to approximate the activity of premature infant liver and thus obtain minimal

TABLE IV

The effect of additions of various fractions on tyrosine oxidation

Subject	Material added	Soluble fraction $\mu l.\ O_2/30'/ml.$
I. Premature infant		
P—14		24
P—14	Supernate of boiled soluble fraction of rat liver	15
P—16		3
P—16	Ultrafiltrate of soluble fraction of rat liver	3
——	Ultrafiltrate of soluble fraction of rat liver	0
II. Adurltat		
AR—16		324
AR—16	Soluble fraction of premature infant liver	294

biochemical differences. The biochemical values obtained with the premature infant liver were more significant since distinctly higher values were obtained with the liver of an adult having had a disease which led to severe parenchymatous disruption. This approach again obviates the criticism that premature infant liver was more disrupted by autolysis after death than normal adult liver. In this analysis (Table V) all of the known intermediates were determined with a combination of various methods. The concentration of p-hydroxyphenylpyruvic acid was determined following incubation of the soluble fraction in an atmosphere of nitrogen (.04 mg. per ml. for premature infant liver and .17 mg. per ml. for adult liver) and the presence of this substance was confirmed in both instances by the subsequent formation and isolation of the 2–4 dinitrophenylhydrazones. When other samples of the same tissue were incubated in air there was a trace of 2–4 dinitrophenylhydrazone formed after the incubation of the premature infant liver and none after adult liver. These results, together with the identity of the results obtained with the Briggs' reaction (.08 mg. per ml.) and with iodometry (.07 mg. per ml.), established that in the case of the adult liver the Briggs' reacting material was all homogentisic acid. On the other hand, only

TABLE V

Protocol of a complete experiment using the soluble fraction of human liver

Determination	Substance determined	Premature infant liver		Adult liver	
		In air	Under nitrogen	In air	Under nitrogen
Oxygen uptake $\mu l./30'/ml.$		1	0	28	0
Millon test* mg./ml.	Tyrosine + p-Hydroxyphenyl-pyruvic acid	1.52	1.55	1.47	1.64
Briggs' test* mg./ml.	p-Hydroxyphenyl-pyruvic + Homogentisic acid	.08	.04	.08	.17
Iodometry* mg./ml.	Homogentisic acid	.02	0	.07	0
Spectrophotometry* ($\lambda 320$)	Maleylacetoacetic acid	+	−	++	−
Dinitrophenyl-hydrazones	p-Hydroxyphenyl-pyruvic acid	±	+	−	+
Nitrogen mg./ml.		4.67		4.25	

* All determinations done after 60 minutes of incubation.

a part of the Briggs' reacting material in the case of the premature infant liver could be accounted for by iodometry (.02 mg. per ml. leaving approximately .06 mg. per ml. by difference). When the premature infant liver and adult liver were incubated in air, the end-product maleylacetoacetic acid was detected in both cases. A greater amount of this substance was present when the adult liver was used. The lack of homogentisic acid (iodometry) and maleylacetoacetic acid (spectrophotometric analysis) when the samples were incubated in nitrogen proved the presence of an anaerobic environment. Thus, by the composite use of various methods the various individual steps in the oxidation of tyrosine were ascertained, and the differences between the premature infant liver and the adult liver were emphasized.

DISCUSSION

These studies show that the liver of the premature infant has little or no tyrosine oxidizing activity. The activity of the tyrosine oxidizing system is greater in the liver of the newborn infant and notably greater in the liver of the adult. These differences appear to be real and apparently do not depend on the conditions of obtaining specimens as indicated by the rat autopsy model and verified by the finding of a very low oxidative activity in the liver of the fetal rat (17).

The premature infants in this study were younger and smaller than the subjects in the previous clinical studies (8) and the *in vitro* activity of fractions of their livers was not stimulated by the addition of ascorbic acid or dichlorophenolindophenol. The addition of ultrafiltrates and boiled supernates of adult rat liver did not activate the liver of the premature infant nor did the soluble fraction of the liver of the premature infant inhibit the activity of the liver of the adult rat. These experiments established the absence of non-protein inhibitors and the presence of the non-protein activators in the liver of the premature infant. A highly significant feature of this study is the demonstration that the activity of the tyrosine oxidizing system increased at a greater rate from prematurity to adulthood than the concomitant increases in cell nitrogen or for that matter any other cellular standard of reference. The over-all relationship of enzyme development to systemic development and differentiation has been well summarized by Boell (26) and Needham (27) who have pointed out that the development of such molecules as enzymes patterns morphologic development. Boell (26) states that enzymes with general and vital functions apparently develop first and keep pace with cell growth whereas enzymes with more specific and less vital functions develop more slowly and later accelerate at a faster rate.

The data previously presented indicate that the low activity of the tyrosine oxidizing system in the liver of the premature infant is not due to a lack of coenzymes or non-protein activators but to the actual absence of apoenzyme. Table V shows that blockage with nitrogen gas at the first step of tyrosine oxidation, using equivalent quantities of tissue nitrogen and an excess of α-ketoglutarate and pyridoxal phosphate, resulted in the production of considerably less p-hydroxyphenylpyruvic acid by premature (.04 mg. per ml. tissue) than adult livers (.17 mg. per ml. tissue). This transamination was studied more completely in rats (17) and it was similarly observed that the tyrosine transaminase apoenzyme was in much lower concentration in the liver of fetal rats than in the liver of adult rats. Transaminase activity may possibly reflect the activity of each of the individual constituent enzymes of the entire enzyme system.

No definitive evidence is at present available to explain the apparent discrepancy between these results and the previous clinical observations (8, 9). One may assume that early in the life of the premature infant only a small amount of tyrosine is converted to p-hydroxyphenylpyruvate due to the paucity of the tyrosine transaminase apoenzyme. This small quantity of p-hydroxyphenylpyruvate would require only a meager concentration of p-hydroxyphenylpyruvate oxidase for oxidation and the apoenzyme could be activated completely by minimal stores of intrinsic ascorbic acid. The above discrepancy could then be readily explained by the further assumption that as the premature infant develops more apoenzymes are synthesized and extrinsic ascorbic acid is necessary to activate the increased p-hydroxyphenylpyruvate oxidase apoenzyme. When this cofactor is not given to premature infants on a high protein diet, the tyrosine oxidizing system is blocked (8, 9) at the stage of conversion of p-hydroxyphenylpyru-

vate to homogentisate; and tyrosine and p-hydroxyphenylpyruvic acid appear in the urine. Observations on premature infants are in progress to determine the *in vivo* and *in vitro* veracity of this theory.

The inconsistently beneficial effect of folic acid on the hydroxyphenyluria of premature infants (14), monkeys (13), and guinea pigs (12) may depend on augmented nonspecific protein synthesis or even on synthesis of the specific apoenzymes. In either case there would result a greater utilization by anabolism or catabolism, respectively, of the substrate presented, *i.e.*, tyrosine. The mechanism or mechanisms involved in the reduction of tyrosyluria by the administration of ACTH is being studied in animals.

SUMMARY

1. The activity of the tyrosine oxidizing system of the liver of the human adult is 10 to 30 times greater, and the activity of the liver of the full-term infant 3 to 5 times greater, than the hepatic activity of the premature infant. These marked differences were consistent, irrespective of the standard of reference used.

2. The *in vitro* addition of large amounts of ascorbic acid did not stimulate the oxidative activity in premature infant liver in contrast to its stimulating effect on adult liver.

3. Premature infant liver does not lack thermolabile or thermostable non-protein activators nor does it contain non-protein inhibitors of the tyrosine oxidizing system.

4. A protocol is presented in which each intermediate of the tyrosine oxidizing system was studied. The evidence suggests that the premature infant in the first few days after birth lacks apoenzyme and that the apoenzyme subsequently develops at a rate in excess of systemic cell or tissue growth.

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THE ACUTE EFFECTS IN MAN OF A RAPID INTRAVENOUS INFUSION OF HYPERTONIC SODIUM BICARBONATE SOLUTION. II. CHANGES IN RESPIRATION AND OUTPUT OF CARBON DIOXIDE[1]

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It is generally agreed that in metabolic acidosis respiration is stimulated, with consequent reduction of alveolar and arterial CO_2 pressure, P_{CO_2}; in metabolic alkalosis, however, there has been disagreement as to whether or not there is a corresponding respiratory inhibition, with elevation of P_{CO_2}. Evidence that such inhibition does occur has been presented in several reports (1–4); on the other hand, some investigators have found no significant change in P_{CO_2} (5). Opportunity for the collection of further evidence on this question was afforded by a series of studies on the effects of acute metabolic alkalosis induced in human subjects by the infusion of hypertonic sodium bicarbonate solution, approximately 2.3 mEq. per kilogram of body weight. The first paper of this series (6) described the ionic transfers as observed in arterial or cutaneous blood, and calculated for extracellular and other body fluid compartments, including the volume of distribution of the excess sodium bicarbonate. The plasma acid-base data from these experiments readily permit the calculation of arterial P_{CO_2}, before, during, and for two hours following the infusion. We consider the changes in calculated arterial P_{CO_2} to represent the most reliable index of respiratory changes, because this sampling method produced the least disturbance to respiration. However, we are also reporting in this, the second paper of the series, results of additional experiments in which alveolar P_{CO_2}, total pulmonary ventilation, and respiratory output of CO_2 were measured. The effects of the infusion on urinary electrolyte excretion and renal hemodynamics are presented in a final paper (7).

Our results indicate that respiration was stimulated during, and depressed following the infusion. This biphasic respiratory response distinguishes sodium bicarbonate from other alkalinizing solutions, such as sodium carbonate and sodium hydroxide. The latter solutions, when given intravenously to dogs, have been shown to produce respiratory inhibition both during and after the infusion (1, 4). It will be demonstrated that the initial stimulation of respiration by sodium bicarbonate occurs only during the infusion and is associated with an increased output of CO_2.

METHODS

The subjects, all men, were volunteers in good health or convalescent hospital patients. All tests were carried out with the subject supine, at rest, and shielded as far as possible from distracting procedures that might stimulate respiration through apprehension or discomfort. The venipuncture required for the administration of the sodium bicarbonate solution was made 10 minutes or more prior to the actual infusion period, the needle being kept open with a slow drip of 5 per cent glucose in water. A solution of 0.89 molar (7.5 per cent) sodium bicarbonate was used,[4] with a total dose ranging from 1.36 to 2.50 mEq. per kilogram of body weight, the average for most subjects being close to 2.3 mEq. per kg. The entire volume of this solution, usually 150 to 200 ml., was infused in 10 minutes. No untoward effects were observed as a result of this dose and infusion rate; some subjects noted minor symptoms, such as transient tingling of the skin or a sensation of cold in the infusion arm.

In the experiments involving arterial blood, an indwelling needle was inserted into the femoral artery 30

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[4] A supply of the sterile solution in ampule form was generously furnished by the Abbott Laboratories.

to 90 minutes before the first control sample was taken, to allow ample time for the subject to assume a stable level of quiet respiration. The needle was connected via a plastic tube with a stopcock manifold, to which were attached the sampling syringes and a wash-out syringe containing a saline-heparin solution. Each sampling syringe was prepared with a measured amount of the heparin solution to eliminate air-containing dead space and serve as an anticoagulant. Blood samples were stored in the syringes, in a beaker of cracked ice, until samples were withdrawn for centrifuging or pH determination. The handling of blood for centrifuging and separation of plasma was accomplished by a "virtual anaerobic" technic (8). In this technic the use of oil or paraffin is avoided entirely, at the expense of about 1 in 15 seconds' exposure of the sample to air during the filling of the centrifuge tube or withdrawal of the plasma sample. The effect of such exposure on pH and plasma CO_2 content is extremely small (8): a mean decrease of 0.005 for the former and 0.1 mM per L. for the latter. The resultant effect on calculated arterial P_{CO_2} is nil.

The total CO_2 concentration of plasma was measured manometrically (9); pH of "whole blood" (actually, plasma pH) was determined either by a photocolorimetric method (8) in the Klett-Summerson filter photometer, or by means of a Cambridge Research Model glass electrode and pH meter. Readings were carried out at 37° C. (photocolorimetric), or at room temperature (glass electrode), with Rosenthal's correction of −0.0147 per degree Centigrade temperature difference to obtain pH at 37° C. (10). The validity of the colorimetric pH determination or its photometric adaptation appears to be well substantiated for human blood or plasma when the readings are made at 37° to 38° C. (8, 11–13). In our hands the standard error of the mean photocolorimetric pH in triplicate 0.1-ml. aliquots was ± 0.0051, and of the mean glass electrode pH in duplicate 1-ml. aliquots (employed only in two experiments), approximately ± 0.01. The standard error of the mean plasma CO_2 determination, for duplicate, 0.2-ml. aliquots, was ± 0.11 mM per L. Chemical methods for factors other than pH and CO_2 content were the same as those described previously (6).

From the pH and plasma total CO_2 concentration the P_{CO_2} was calculated by the Henderson-Hasselbalch equation, in which pK′, was assigned the value 6.10, and f_{CO_2}, the solubility factor for dissolved CO_2 gas plus carbonic acid at 37° C. in plasma, the value 0.0314 mM per L. per mm. P_{CO_2} (13–16):

$$pH = pK_1' + \log \frac{(CO_2)_p - f_{CO_2}P_{CO_2}}{f_{CO_2}P_{CO_2}}$$

These acid-base data, in conjunction with the hemoglobin concentration, were also used to obtain the whole blood buffer base concentration by means of a nomogram devised for that purpose (17). The buffer base, 49 ± 3 mEq. per L. in normal blood, is the cation equivalent to the sum of all buffer anions and is a convenient index of the metabolic or electrolyte factor in the acid-base balance of blood, independent of the chemical effects of changing respiration and P_{CO_2}.

Nine sodium bicarbonate experiments were carried out in which arterial blood was sampled as described above (Group A). Three similar control experiments (Group D) were performed with 5 per cent glucose, 0.9 per cent sodium chloride, or 25 per cent mannitol substituted as a neutral solution in place of the sodium bicarbonate. Respiratory measurements were carried out in two other groups of subjects. End-expiratory "alveolar" air was collected from 5 subjects (Group B) by means of a semi-automatic sampling device (18) and analyzed for CO_2 with the Scholander gas analyzer (19); three of these were carried out in combination with arterial blood sampling. Expired air collections were made in another group of 5 subjects (Group C), and data were obtained for respiratory rate, tidal volume, total ventilation, and output of CO_2. To a large extent respiratory measurements were avoided in the Group A subjects, because

TABLE I

Subject S. R.—arterial blood and alveolar air data *

Sample	Time†	Observed data: Arterial blood: Hemoglobin	Cell volume	pH	Plasma CO_2	Plasma Na	Plasma Cl	Alv. air: P_{CO_2}	Calculated: Arterial blood: P_{CO_2}	$(BB^-)_b$‡
	min.	*mM/L.*	*%*		*mM/L.*	*mEq./L.*	*mEq./L.*	*mm.*	*mm.*	*mEq./L.*
1	−20	9.3	47.5	7.38	27.7	142	104.6	41.7	45	49.0
2	−16	9.2	47.5	7.37	27.7	141	107.3	44.0	46	48.5
3	− 7	8.6	45.0	7.44	34.8	144	103.9	47.2	50	55.5
4	− 0.5	8.5	44.5	7.48	38.5	146	103.0	47.0	51	60.0
5	+ 5	8.8	43.0	7.44	35.3	145	101.9	46.7	51	56.0
6	+10	8.8	43.0	7.43	34.0	142	104.6	46.2	50	55.0
7	+22	9.0	43.0	7.41	32.7	142	103.1	46.3	50	53.0
8	+60	9.0	44.5	7.40	31.5	142	103.1	46.2	49	52.5

* IV infusion of 156 mEq. of $NaHCO_3$ (175 ml. of 7.5 per cent solution) from −11.5 to 0 minutes.
† Time referred to end of infusion as 0 minutes.
‡ Whole blood buffer base concentration.

TABLE II

Subject S. R.—respiratory data (second infusion) *

Period	Time†	Mean respiratory Rate	Tidal vol.	Total vent.	Expired air CO_2 content	Rate of CO_2 elim.	
	min.	*per min.*	*ml.*‡	*L./min.*‡	%	*ml./min.*§	*mM/min.*
1	−68.0 to −60.0	9.7	525	5.1	4.13	178	8.0
2	−49.0 to −41.0	11.7	490	5.7	3.57	174	7.8
3	−25.0 to −16.5	10.8	460	5.0	3.90	166	7.5
4	− 8.0 to 0.0	11.5	535	6.2	4.61	240	10.8
5	+ 3.0 to +11.0	9.2	520	4.8	4.31	175	7.9
6	+14.0 to +22.0	8.2	570	4.7	4.47	177	8.0
7	+49.0 to +57.0	8.2	580	4.8	4.72	190	8.5

* IV infusion of 156 mEq. of $NaHCO_3$ (175 ml. of 7.5 per cent solution) during period 4.
† Time referred to end of infusion as 0 minutes.
‡ ATPS (758 mm. and 26.5° C.).
§ STPD.

the necessary sampling procedures would have disturbed the base-line respiration and thus vitiated the unique advantage of the arterial technic.

RESULTS

Complete data for a single subject, S. R., on arterial blood and alveolar air are given in Table I; similar respiratory measurements are shown in Table II. These are representative detailed results from the three groups of experiments, A, B, and C.[5] The maximum acid-base changes in arterial blood are seen to occur at the end of the infusion: rise in pH, whole blood buffer base, and concentrations of sodium, total CO_2, and bicarbonate in plasma. These changes characterize a primary metabolic alkalosis of the sodium excess type. The fall in hemoglobin and plasma chloride concentrations was a dilution effect, due to the volume of the infused solution and its hypertonic character, as a result of which water was drawn from tissue cells. There was an initial rapid recovery rate in the first half-hour, and a slower one thereafter. We have estimated that at the end of two hours an average of 51 per cent of the dose can still be accounted for in red cells and "extracellular fluid," 27 per cent has been excreted in the urine, and the remaining 22 per cent has apparently penetrated "intracellular fluid" (6). The maximum rise in calculated arterial and alveolar P_{CO_2} also occurred at the end of the infusion. The total ventilation rose during the infusion and subsequently fell. The rate of pulmonary CO_2 output was sharply elevated during the infusion, then returned to its pre-infusion level.

The individual curves for each subject, calculated as serial differences from the mean control levels of pH, P_{CO_2}, and respiratory factors, are given in Figures 1 to 3. The response of the 9

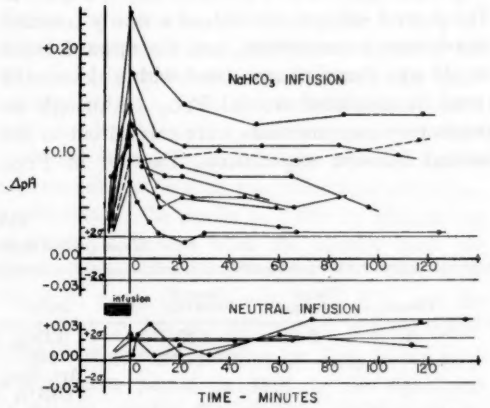

FIG. 1. CHANGES IN ARTERIAL BLOOD pH FROM MEAN CONTROL LEVELS EXHIBITED BY NINE NORMAL SUBJECTS FOLLOWING THE INFUSION OF HYPERTONIC SODIUM BICARBONATE (UPPER PORTION OF GRAPH) AND SIMILAR CHANGES EXHIBITED BY THREE NORMAL SUBJECTS FOLLOWING THE INFUSION OF NEUTRAL SOLUTIONS (LOWER PORTION OF GRAPH)

Mean control values are given in Table III. The dashed curve represents subject R. L., who had very low control pH values.

[5] Nine of the experiments in Groups A and D were continued for the full 2 hours after the infusion. Complete arterial blood data for 6 other subjects in Group A may be found in the preceding paper (6).

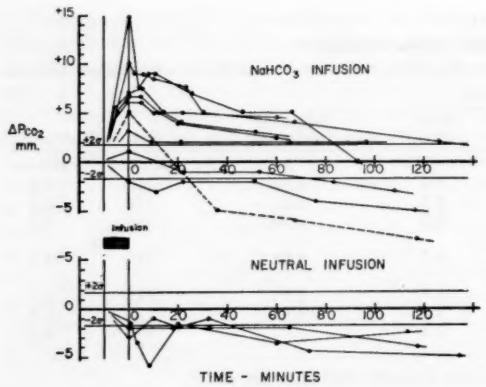

FIG. 2. CHANGES IN CALCULATED ARTERIAL P_{CO_2} FROM MEAN CONTROL LEVELS EXHIBITED BY NINE NORMAL SUBJECTS FOLLOWING THE INFUSION OF HYPERTONIC SODIUM BICARBONATE (UPPER PORTION OF GRAPH) AND SIMILAR CHANGES EXHIBITED BY THREE NORMAL SUBJECTS FOLLOWING THE INFUSION OF NEUTRAL SOLUTIONS (LOWER PORTION OF GRAPH)

Mean control values are given in Table III. The dashed curve represents subject R. L., who had very high control P_{CO_2} values.

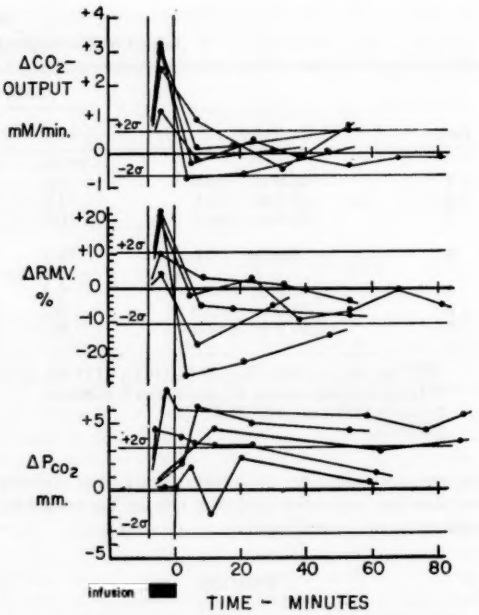

FIG. 3. CHANGES FROM CONTROL VALUES EXHIBITED BY GROUPS OF FIVE NORMAL SUBJECTS FOLLOWING THE INFUSION OF HYPERTONIC SODIUM BICARBONATE, WITH RESPECT TO RESPIRATORY ELIMINATION OF CO_2 (UPPER), RESPIRATORY MINUTE VOLUME (MIDDLE), AND END-EXPIRATORY, "ALVEOLAR" P_{CO_2} (LOWER)

Mean control values are given in Table III.

subjects of Group A is compared with that of the 3 subjects receiving neutral infusion, with respect to pH in Figure 1, and arterial P_{CO_2} in Figure 2. The control subjects maintained a nearly constant bicarbonate concentration, and the upward trend in pH was therefore associated with a downward trend in calculated arterial P_{CO_2}. Although no respiratory measurements were carried out in the neutral infusion experiments, the fall in P_{CO_2} implies an increase in total ventilation. In the sodium bicarbonate experiments the rise in alveolar P_{CO_2}, the transient rise in CO_2 output during the infusion, and the generally biphasic re-

TABLE III

Mean control data and dose of $NaHCO_3$

Group	No. of subjects	Dose of $NaHCO_3$	Factor	Unit	Mean	S.D.
A	9	2.29 mEq./Kg.	$(CO_2)_p$	mM/L.	26.8	±0.90
			pH		7.37*	0.036*
			Art. P_{CO_2}	mm. Hg	45.0†	3.68†
			$(BB^+)_b$	mEq./L.	47.7	1.61
B	5	2.15	Alv. P_{CO_2}	mm. Hg	39.9	2.81
C	5	1.61	Tot. vent.	L./min. ATPS	6.28	1.19
			CO_2 output	mM/min.	9.15	0.88
D	3	0 (Neutral infusions)	$(CO_2)_p$	mM/L.	27.8	1.90
			pH		7.37	0.015
			Art. P_{CO_2}	mm. Hg	46.2	1.02
			$(BB^+)_b$	mEq./L.	48.0	1.95

* Omitting subject R. L., pH 7.38 ± 0.023.
† Omitting subject R. L., arterial P_{CO_2} 43.9 ± 1.38.

TABLE IV

Significance of mean changes from control levels

Interval, control per. to period of	Factor	Group A	Group B	Group C		Group D*
		Art. ΔP_{CO_2}	Alv. ΔP_{CO_2}	ΔTot. vent.	ΔCO_2 output	Art. ΔP_{CO_2}
		mm. Hg	*mm. Hg*	*% control*	*% control*	*mm. Hg*
Infusion	Mean diff.	*+4.5*‡	+4.0	*+14*	*+29*	
	S.E.	±0.29	±2.25	± 3.70	± 4.78	
	t = Δ/S.E.	15	1.78	3.78	6.06	
	D.F.	2	2	4	4	
	P†	<0.01	0.3	0.02	<0.01	
Early post-infusion (0 to 55 min.)	Mean diff.	*+3.5*	*+3.8*	−9.3	0	*−2.3*
	S.E.	±1.25	±0.95	±3.62	±4.61	+0.41
	t = Δ/S.E.	2.80	4.05	2.60	0	5.72
	D.F.	8	4	4	4	2
	P	0.03	0.02	0.06	not sign.	0.04
Late post-infusion (55 to 127 min.)	Mean diff.	+0.3				*−3.7*
	S.E.	±1.21				±0.62
	t = Δ/S.E.	0.25				5.97
	D.F.	8				2
	P	>0.5				0.03

* Group D—received neutral infusion instead of $NaHCO_3$.
† P values taken from "Student's" t test, for calculated value of t and number of degrees of freedom, D.F.
‡ Significant differences ($P \leqslant 0.05$) italicized.

sponse of total ventilation (results from Groups B and C) are shown in Figure 3. The horizontal lines above and below the base-line of 0 change in Figures 1 to 3 represent 95 per cent confidence limits of the intra-individual variability. For each factor the standard deviation, σ, of all control observations about the respective individual means was calculated. Confidence limits were set at $\pm 2\sigma$, and were found to be ± 0.46 mM per L. for plasma CO_2 concentration, ± 0.022 for pH, ± 1.72 mm. of Hg for calculated arterial P_{CO_2}, ± 0.80 mEq. per L. for whole blood buffer base, ± 3.22 mm. of Hg for alveolar P_{CO_2}, ± 0.78 L. per min. ATPS for total ventilation, and ± 0.54 mM per min. for CO_2 output. The control observations of Groups A and D were pooled to give a common set of confidence limits.

It is evident from Figures 1 to 3 that the changes in arterial pH and CO_2 output were consistent in all sodium bicarbonate experiments, and uniformly significant on the basis of the confidence limits of control variability. However, in a minority of tests the P_{CO_2} was not significantly elevated, and the total ventilation was not significantly depressed following the infusion. In the second hour after the infusion, 3 of the subjects in Group A exhibited a significant *decrease* in arterial P_{CO_2} (Figure 2). This response is similar to the one shown by the subjects receiving neutral infusions. The dashed curve in Figures 1 and 2 is that of a subject having the extremely high control P_{CO_2} of 54 mm. of Hg.

The significance of mean changes in the various groups was evaluated by the usual t test, the control values being compared with those obtained (a) during the infusion, (b) during the early post-infusion period, from 0 to 55 minutes after the end of the infusion, and (c) during the late post-infusion period. Table III contains mean values, with standard deviation, for the respiratory and acid-base factors in each of the four groups of experiments, as well as the mean dose of sodium bicarbonate. The results in Table IV include sets of mean differences from the control level, the standard error of each difference, S.E., the t value (ratio of mean difference to its S.E.), and the probability, p, that the difference is due to chance. The rise in arterial and alveolar P_{CO_2} in the early post-infusion period is seen to be significant ($p \leqslant 0.05$); during the infusion only the arterial P_{CO_2} was significantly elevated, but the number of observations was small. The 14 per cent elevation of total ventilation during the infusion was significant, but the 9.3 per cent decrease in the early post-infusion just failed to attain the 95 per cent level of significance with a p of 0.06. The increase in CO_2 output during the infusion was highly significant, and the mean change was nil for the early

post-infusion period. In the group of subjects given neutral infusions the arterial P_{CO_2} was significantly decreased in both the early and late post-infusion periods.

DISCUSSION

These results exhibit the biphasic character of the respiratory response to sodium bicarbonate infusion in man: an initial increase in total ventilation, indicating a stimulation to respiration, during the infusion; and a decrease in total ventilation, indicating respiratory inhibition, following the infusion. This biphasic character is a unique feature of sodium bicarbonate administration; other alkalinizing solutions, such as sodium hydroxide and sodium carbonate, evoke a decrease in total ventilation both during and after the infusion (1, 4). The initial stimulation of respiration by sodium bicarbonate solutions has been reported in experiments on dogs by Gesell, Krueger, Gorham, and Bernthal (1), Bernthal (3), and Hesser (4).

The difference in action of sodium bicarbonate as compared with other alkalinizing solutions is related to the chemical reactions that occur in blood during the infusion. Highly alkaline solutions produce a relatively marked rise in pH and transient fall in H_2CO_3 and therefore the P_{CO_2} of admixed blood:

$$Na^+ + OH^- + HBuf \rightarrow Na^+ + Buf^- + H_2O$$
$$Na^+ + OH^- + H_2CO_3 \rightarrow Na^+ + HCO_3^- + H_2O$$

The symbol Buf^- represents non-bicarbonate buffer anion, chiefly hemoglobin and plasma protein anion, and HBuf the corresponding undissociated buffer acid.[6] In the case of the rather weakly alkaline sodium bicarbonate solution, most of the added Na^+ and HCO_3^- ions simply increase the initial concentrations of these ions in blood. However, to some extent the following reactions occur, the dehydration of H_2CO_3 being catalyzed by the enzyme carbonic anhydrase in red cells:

$$Na^+ + HCO_3^- + HBuf \rightarrow Na^+ + Buf^- + H_2CO_3$$
$$H_2CO_3 \xrightarrow[\text{anhydrase}]{\text{carbonic}} H_2O + CO_2\uparrow$$

[6] In actual fact, there is little penetration of excess Na^+ with OH^- or HCO_3^- into the red cell. Exchange of plasma HCO_3^- for a smaller amount of cell Cl^- has the same effect in permitting the red cell $HHbO_2$ to carry out its share of the buffering action.

The vertical arrow symbolizes the volatile nature of the excess CO_2 thus formed, since, under ordinary circumstances, it will bubble off from a blood-mixture in a beaker, or appear as non-metabolic CO_2 in expired air. This is the explanation for the rise in CO_2 output in expired air during the infusion period (Figure 3). For each millimole of excess H_2CO_3 or CO_2 produced in this reaction a milliequivalent of buffer anion, Buf^-, is also formed. It is therefore possible to calculate the quantity of non-metabolic CO_2 appearing in expired air during the infusion period from the increase in Buf^-, which can, in turn, be calculated from the pH rise, the titration properties of human blood (16), and the estimated quantity of non-bicarbonate buffers in the whole blood volume, as described in the preceding paper (6). We have made a calculation of this type for the mean result of the Group C experiments, recognizing that it is an approximation only, since blood studies were not carried out simultaneously in the subjects of this group. The mean ΔpH for the nine experiments of Group A was + 0.14, corresponding to a mean dose of 2.29 mEq. per kg. of body weight. The value of ΔpH estimated for the mean of the five subjects of Group C, with a smaller dose of 1.61 mEq. per kg., would therefore be + 0.10. The slope of the titration curve of normal human blood with a hematocrit value of 45 per cent averages 25 mEq. per L. per pH unit (16, 17). The blood volume was assumed to be 80 ml. per kg. of body weight (20), or a mean of 6.2 liters for these subjects (average weight 78 kg.). The rise in non-bicarbonate buffer anions, ΔBuf^-, was thereby calculated:

$$\Delta Buf^- = (0.10)(25)(6.2) = 16 \text{ mEq.}$$

The mean quantity of excess CO_2 collected during the infusion period was 18 mM for the same five subjects, a close agreement, considering the number of assumptions made in the calculation.

Any attempt to postulate a mechanism for the respiratory stimulation during sodium bicarbonate infusion leads to manifold and complex considerations that lie beyond the scope of this paper. Available evidence reveals only two points of difference between the stimulation seen during infusion of sodium bicarbonate and the inhibition during the infusion of other alkalinizing solutions: the

rise in CO_2 output and the rise in P_{CO_2} of *venous blood,* at least locally where blood is mixed with sodium bicarbonate infusion, as contrasted with the decrease in these variables when non-bicarbonate alkalinizers are administered by vein. The P_{CO_2} of *arterial blood* (supplying both the carotid body chemo-receptors and the cells of the respiratory center) is elevated regardless of the type of alkalinizing solution being infused.

In the first hour after the end of the infusion our observations conform to the usual concept of secondary respiratory inhibition in any primary metabolic alkalosis: decrease in total ventilation and increase in arterial and alveolar P_{CO_2}, associated with the rise in blood pH, and bicarbonate and buffer base concentrations. Once it has been established that the CO_2 output of expired air does not differ from the control level (Table IV), a significant elevation of calculated arterial P_{CO_2} must be associated, through the alveolar equation, with a significant fall in alveolar ventilation, hence a respiratory inhibition. We regard this as sufficient evidence for a significant average respiratory response, even though the observed decrease in total ventilation was found to have a p value between 0.05 and 0.06.

Although satisfactory levels of significance were established for the changes of respiratory inhibition in the first hour after the infusion, there was a wide scatter of individual response in the second hour. In some individuals with primary metabolic alkalosis ventilation is not decreased, and the P_{CO_2} of arterial, or its equivalent, cutaneous blood is not elevated. The problem is further complicated when clinical patients are studied: control observations are usually not available, and the upper limit of normal P_{CO_2} appears to be higher than generally supposed, if physiological variations and truly basal conditions of respiration are taken into account (Table III, references 21–23). Even if the too low conventional limit of 45 mm. of Hg is used as this dividing line in arterial or cutaneous blood, the incidence of P_{CO_2} values exceeding 45 mm. is found to be only 26 per cent in one series of patients with metabolic alkalosis (24). Thus it is not surprising that the existence of a secondary respiratory inhibition in this acid-base disturbance should have been denied by West and Rapoport (5). However, the experimental evidence of these authors, obtained on three dogs, is open to the criticism that conclusions were based on the analysis of venous blood, in which the P_{CO_2} is a much less reliable index of respiration than it is in arterial or cutaneous blood. At least one of the papers cited by West and Rapoport in support of their view actually confirms the presence of a respiratory inhibition. This is the work of Shock and Hastings, involving serial cutaneous blood observations up to seven hours after the oral ingestion of sodium bicarbonate by normal human subjects (2). From supplementary protocols, referred to but not printed in full in Shock and Hastings' paper, we have calculated that the mean rise in P_{CO_2} was highly significant ($p < 0.01$) in nine such experiments, during the period from one-half to three hours after the maximum effect on blood bicarbonate.

Other factors that contribute to the difficulty of detecting and measuring such a small inhibitory effect are the ease with which respiration is stimulated by psychic and other extraneous stimuli (25, 26), and the influence of the experimental sampling procedure on respiration. The effect of both factors is almost invariably to stimulate respiration, increase total ventilation, and thus lower arterial P_{CO_2}. In experimental tests this influence is mediated through apprehension and discomfort incident to the sampling procedure or other aspects of the experiment, or through the increase in physiological dead space introduced by most gas sampling devices that do not require the cooperation of the subject. As confirmed in trials of our own, the alveolar P_{CO_2} of subjects breathing naturally is higher than it is when expired air or alveolar air is collected by such devices. Even with the Haldane-Priestley method of sampling alveolar air respiration is disturbed by the forced expiration for as long as 10 minutes. These were the considerations that led us to select the calculated arterial P_{CO_2} as the principal index of respiratory activity, because the arterial blood samples could be obtained at will, without any detectable disturbance to the subject.

Despite these interfering factors tending to stimulate respiration, it was possible to demonstrate a significant inhibitory response for a group of normal subjects under our conditions of experimental metabolic alkalosis. A similar response has been found by others in some but not

all individuals with this type of acid-base disturbance (*2, 24, 27, 28*). We therefore regard decrease in total ventilation and elevation of P_{CO_2} as the characteristic secondary changes in any kind of metabolic alkalosis, clinical or experimental. Failure to observe such an inhibition may be due to extraneous stimuli, to the special circumstance of infusing sodium bicarbonate solution, or to the coexistence of a primary respiratory alkalosis, such as might be seen in a dyspneic patient with congestive heart failure, given prolonged treatment with mercurial diuretics (*29*).

SUMMARY

1. In different groups of normal human subjects observations were made before, during, and after the infusion of hypertonic sodium bicarbonate solution, of the following acid-base and respiratory factors: (a) arterial CO_2 pressure, P_{CO_2}, as calculated from pH and plasma CO_2 concentration; (b) end-expiratory alveolar P_{CO_2}; (c) total ventilation, from collection of expired air; (d) rate of elimination of CO_2 in expired air.

2. *During* the infusion of sodium bicarbonate, arterial pH, arterial and alveolar P_{CO_2}, total ventilation, and rate of elimination of CO_2 were significantly increased above control levels. These findings indicate both respiratory stimulation and the intravascular formation of excess carbonic acid, by reaction of the infused bicarbonate ion with the relatively acid protein buffers of blood. Of the various solutions which are biologically alkalinizing only bicarbonate salts produce this initial stimulation.

3. *Following* the infusion, the rate of CO_2 elimination returned to the control level, but arterial pH was still elevated despite a steady fall toward the control range. In the first hour after the infusion there was a significant elevation of mean arterial and alveolar P_{CO_2} in the groups tested. The corresponding decrease in mean total ventilation was not significant at the 5 per cent level but was significant at the 6 per cent level. These findings indicate a significant degree of secondary respiratory inhibition for the conditions of acute metabolic alkalosis obtaining in these experiments.

4. In three normal subjects given neutral infusions instead of sodium bicarbonate, the arterial P_{CO_2} was significantly decreased from control levels in the first two hours after the infusion. Reasons are presented in support of the view that failure to observe the characteristic respiratory inhibition in some individuals with metabolic alkalosis may be attributed to various types of extraneous or psychic stimulation.

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METABOLISM OF SALT-RETAINING HORMONE BY SURVIVING LIVER SLICES[1]

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Evidence has been produced in this and other laboratories that the impaired capacity to excrete sodium which characterizes a number of clinical states involving edema, ascites and anasarca, is mediated, in part at least, through a humoral factor emanating, in all probability, from the adrenal cortex (1–8). It now appears almost certain, on the basis of available evidence, that this substance, generally referred to in this paper as salt-retaining hormone or electrocortin, is aldosterone or a derivative of it (9, 10).

Since quantitative measurements indicate that this sodium-retaining hormone is excreted in the urine in abnormally high concentration under these pathological conditions, curiosity concerning the underlying mechanism naturally arises. Two hypothetical possibilities are suggested: 1. Overproduction of salt-retaining hormone by the adrenal cortex initiates an abnormal anti-natriuretic and antidiuretic response. 2. Physiological inactivation or metabolism of the hormone by extra-adrenal tissues is impaired with a resulting rise in blood, tissue and urine concentrations of "active" hormone. However, it is possible that accumulation of salt-retaining hormone, whatever its cause, bears no cause and effect relationship to fluid retention and other related manifestations of disease.

In view of the recent evidence by Conn (11, 12) and Mader and Iseri (13) consideration must be given to the first of these possibilities. "Primary aldosteronism," a new syndrome described by these investigators, is characterized by hypernatremia and hypokalemia, but not necessarily edema. In this condition an adrenal tumor has been found which appeared to be secreting large quantities of aldosterone. It is doubtful, however, if adrenal cortical hyperfunction of this type can be indicted as a frequent cause of the high levels of sodium-retaining factor found in various pathologic states in which sodium retention and edema are characteristically seen. In toxemias of pregnancy the rapid fall in the urinary titer following delivery would suggest that high aldosterone levels derive from some transient derangement of functional control in contrast to the apparently continuous hyperfunction found in primary aldosteronism. Also, evidence of adrenal cortical hyperfunction is not a consistent finding in hepatic cirrhosis, congestive heart failure and nephrosis, diseases in which salt retention, edema, and high urinary aldosterone levels are characteristic (3, 7, 14). That there is a cause and effect relationship, however, between the abnormal accumulation of fluid and sodium retention in cirrhosis of the liver, congestive heart failure and toxemia of pregnancy is suggested by the now well recognized importance of dietary salt restriction in retarding or preventing edema and ascites in these conditions.

In an attempt to obtain further evidence bearing on the second hypothesis, the capacity for *in vitro* inactivation of this salt-retaining factor by surviving liver slices has been studied, and the results, previously reported in abstract form (15), are presented in this communication.

EXPERIMENTAL

Twenty-four-hour urine collections from patients with cirrhosis of the liver, congestive heart failure and preeclamptic toxemia provided the active salt-retaining material. This urine was stored in the refrigerator without preservative and extracted within twenty-four hours. Extraction with chloroform followed hydrolysis at pH 1 (sulfuric acid) overnight at room temperature, according to a standard procedure previously published

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(2). The final extract, which contains all active adrenal steroid compounds with the exception of estrogens and androgens, was then partitioned in a benzene-water system. Under these conditions, the sodium-retaining activity moves along with cortisone and hydrocortisone into the water phase, while a number of contaminating compounds including a large amount of pigment, are removed in the benzene phase. Most of the extracts were then subjected to partition chromatography in a toluene-propylene glycol system on filter paper according to the general scheme of Zaffaroni and Burton (16). Under these conditions the bands of compounds F and E are widely separated with the latter moving 10 to 12 cm. farther. The papers were then divided into three sections by cutting below the compound F band and below the compound E band, the sections were eluted with an absolute methanol-chloroform mixture and the eluates evaporated to dryness. The eluates were assayed for sodium-retaining activity according to the method of Kagawa, Shipley, and Meyer (17). The active sodium-retaining fraction was consistently found in close association with the cortisone band. Potency was recorded in terms of percentage of sodium retained per unit of extract, under the specific experimental conditions of the assay.

The active extracts of known potency were then dissolved in small amounts of absolute ethanol and incubated with surviving rat liver slices for two hours in 15-ml. Warburg flasks at 37 degrees Centigrade. Krebs-Ringer-phosphate buffer was employed as the incubation medium and the flasks contained potassium hydroxide in the center well. Fifty to sixty-milligram liver slices (wet weight) were used per flask and the active extract in ethanol was prepared so that an adequate dose for five assay animals was contained in 0.1 ml. Respiration was measured and QO_2 values were calculated. Control flasks were prepared containing no tissue, boiled tissue, and to others 0.01 molar cyanide was added. In several instances anaerobic incubation was carried out using bicarbonate buffer in an atmosphere of nitrogen. Kidney slices were also studied by the same technique and, in addition, one experiment was performed with human liver, using tissue obtained at laparotomy from a patient with no evidence of liver or endocrine disease, and without any suggestion of a disorder in either fluid or electrolyte balance.

At the end of the incubation period, contents of the flasks were re-extracted with chloroform, and the resulting extracts re-assayed under experimental condi-

TABLE I

Sodium retaining activity of urine extracts after incubation with rat and human liver slices *

Experiment No. 1 Extract MH	Oil control	Untreat. extract	Liver	Cyanide liver	Boiled liver	No tissue	Kidney	Anaerobic liver	Cyan. No tiss.
Sodium exc. mg.	2.42	1.66	2.53	2.67	1.54	1.74	1.79		
Stand. error	0.23	0.14	0.26	0.70	0.26	0.23	0.23		
"t"	3.04†		3.27†	2.3‡	0.14	0.1	0.53		
Per cent act.		100	0	0	100	90	83		
Experiment No. 2 Extract OS									
Sodium exc. mg.	2.6	0.87	3.35	2.53	1.1	0.81	0.84	1.87	
Stand. error	0.22	0.1	0.34	0.34	0.27	0.13	0.03	0.59	
"t"	8.23†		9.92†	6.26†	0.98	0.29	0.15	2.98†	
Per cent act.		100	0	4	87	100	100	42	
Experiment No. 3 Extract P-83									
Sodium exc. mg.	2.6	0.85	2.3	2.2	1.06	0.71		2.2	2.05
Stand. error	0.22	0.16	0.14	0.34	0.12	0.12		0.24	0.05
"t"	7.74†		6.41†	4.93†	1.21	0.62		6.0†	5.47†
Per cent act.		100	17	23	88	100		23	31

Experiment No. 4 Extract P-83	Human liver	Boiled human liver	Anaerobic human liver
Sodium exc. mg.	2.37	0.76	1.51
Stand. error	0.19	0.1	0.35
"t"	7.6†	0.41	2.46‡
Per cent act.	13	100	62

* The values shown in the "Sodium Excretion" column are the average values in milligrams of sodium excreted by groups of rats receiving urine extracts which had been subjected to the various treatments indicated. Retention or excretion of sodium can readily be determined by comparing any of the values to the corresponding figures for the "oil control" group.

† $P < 0.01$.

‡ $P < 0.05$.

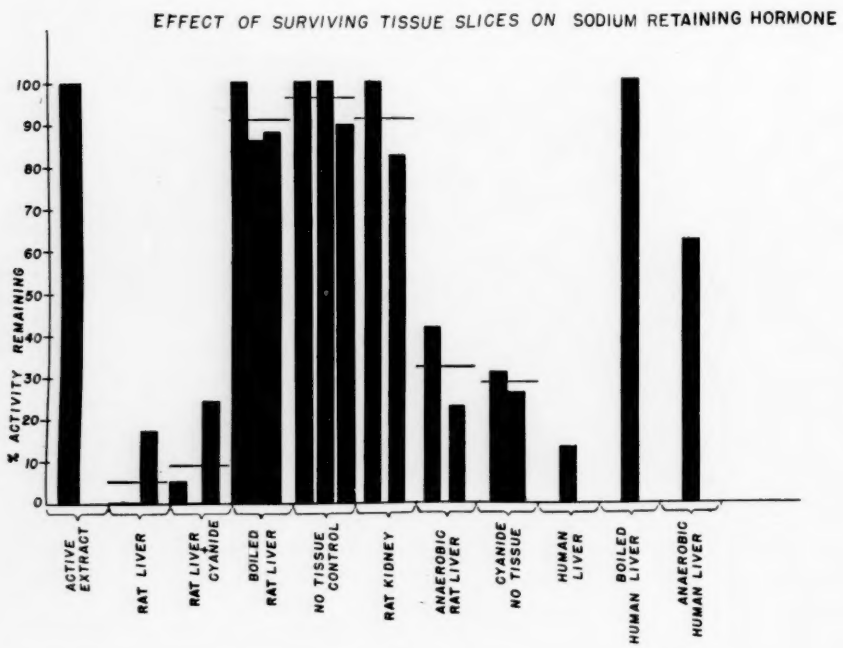

FIGURE 1

tions as nearly identical as possible to the pre-incubation measurements.

RESULTS

Extracts from three different subjects (designated as MH, OS and P-83) were incubated with liver tissue. A summary of results obtained from bioassay of these extracts after incubation is shown in Table I. To test for significant differences between the various treatments, the sodium excretion values were subjected to Fisher's "t" test as described by Snedecor (18). In all cases the "t" values listed for the various groups were obtained by using the sodium excretion found in response to untreated extract as the basis for comparison. In contrast to the oil control animals, the decrease in sodium excretion by animals receiving the untreated extract was highly significant. The "t" values for this comparison are shown in the "Oil Control" column. The relative activity of the sodium-retaining factor after being subjected to the various treatments indicated is tabulated in the "per cent activity" column. The sodium retention exhibited by the untreated extracts is arbitrarily designated as 100 per cent activity, and the retention found in response to the treated extracts is expressed as a percentage of that manifested by the untreated extracts. In some instances it is apparent that more sodium was excreted by groups of animals treated with incubated extracts than was observed in the "Oil control" group. This is interpreted to mean that complete inactivation of the sodium-retaining compound had taken place, but an explanation of the sodium excreting activity of these extracts needs further investigation and lies beyond the scope of this paper. Therefore, in this report, these extracts inducing sodium excretion are considered to contain 0 per cent activity.

Surviving, actively-respiring liver slices caused, in every instance, complete or nearly complete loss of sodium-retaining activity in the post-incubation extracts. Boiled liver slices, on the other hand, produced no significant inactivation. Addition of 0.01 M cyanide invariably produced very great inactivation, which, in some experiments was complete. In the anaerobic system incomplete but significant inactivation was noted with both the rat

and human liver slices. The data suggest that these conditions may impair the inactivating capacity of liver tissue. The decreased inactivation of sodium-retaining factor by human liver incubated anaerobically is statistically significant when compared to aerobic incubation. Kidney slices failed to produce inactivation of importance in two experiments.

Figure 1 presents these data in the form of a bar graph. The activities remaining after the various treatments are calculated as percentages of the corresponding untreated extracts, and are so represented in the histogram. For example, after incubation with normal liver, extracts MH, OS and P-83 show 0 per cent, 0 per cent, and 17 per cent activities, respectively, as compared to their unincubated controls. Mean values are designated by horizontal lines.

DISCUSSION

In common with several other steroid compounds that have been studied, the salt-controlling hormone of the adrenal cortex (considered to be aldosterone) appears to be metabolized and partially inactivated by extra-adrenal tissues, especially the liver. This mechanism has been shown to function in the case of cortisone by Eisenstein (19) and for cortisone and hydrocortisone by Schneider and Horstmann (20), by Conn, Fajans, Louis, and Seltzer (21), by Bongiovanni and Eisenmenger (22) and by Tyler and his associates (23–25). Similar metabolic effects of the liver on estrogens have been demonstrated by Fishman (26), by Heller (27), by Ryan and Engel (28, 29), and by numerous others (30, 31). In the case of testosterone, Wotiz, Lemon, and Voulgaropoulos (32, 33) have reported similar enzymatic transformations. Hepatic inactivation of aldosterone may be regarded, therefore, as an additional instance of a recognized pattern, and, we believe, may represent an important physiological control mechanism which supplements the fluctuating levels of discharge from the adrenal gland to regulate the amount of circulating "active hormone."

The data from this investigation suggest that anaerobic conditions *in vitro* tend to impair somewhat the otherwise high efficiency of hepatic enzymes for inactivation of the salt-retaining hormone, although this action does not appear to be entirely destroyed. It is tempting to speculate concerning the possible role of hypoxia of the liver in congestive heart failure as a contributing factor in bringing about the elevated urinary level of salt-retaining hormone which has already been demonstrated in this disease.

Addition of 0.01 M cyanide to the incubation

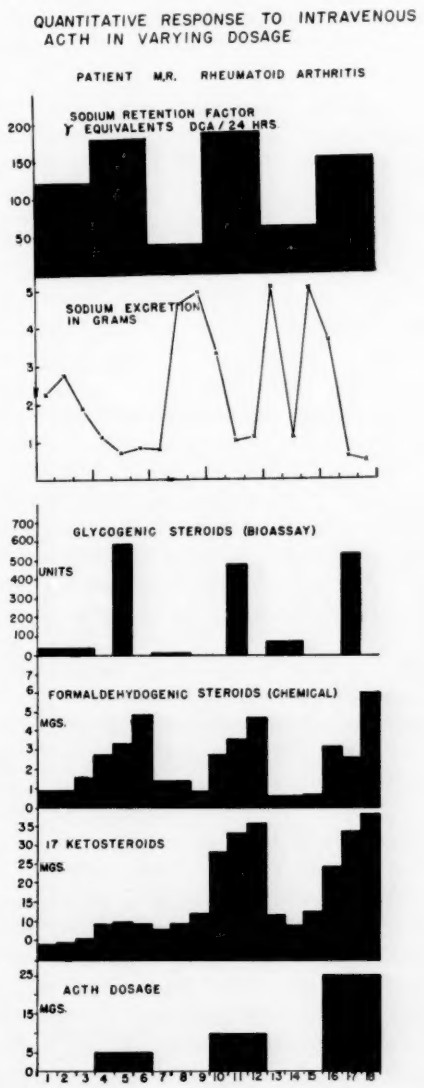

FIGURE 2

preparation, completely inhibited all respiratory activity as anticipated, but the decrease in sodium-retaining potency which followed was nearly as great as that produced by intact respiring liver slices. This result became somewhat clarified when incubation of the extract with cyanide in phosphate buffer, without tissue, also produced approximately the same degree of inactivation. It is apparent that some chemical reaction, between cyanide and the sodium-retaining compound, not dependent upon enzymatic activity, is capable of destroying the biological activity of the steroid hormone.

The means of regulation of adrenal cortical production and release of electrolyte-controlling hormone remain obscure but it appears increasingly clear that it is not entirely under the control of pituitary ACTH, as are the glycogenic corticoids (34, 35). Our own studies, as illustrated in Figure 2, have demonstrated a slight rise in urinary titer of sodium-retaining activity in response to intensive ACTH stimulation, when the hormone is administered by slow intravenous drip over a period of three days. It is quite possible that this small change is related to other steroid compounds produced by the adrenal cortex under ACTH control, such as corticosterone (Compound B). Liddle, Cornfield, Casper, and Bartter (36) have also found the sodium-retaining activity of chromatographed urine extracts of patients receiving ACTH to be increased approximately two-fold and in these experiments, the rise is presumably attributable specifically to aldosterone. In either case, however, these values do not approach the exceedingly high titers that are found so regularly under pathological conditions of salt and water retention. It seems more likely, therefore, that aldosterone release must depend upon total body reserves of sodium and/or potassium, or upon absolute osmolarity of blood and interstitial fluid. It has even been proposed that some other pituitary trophic hormone, as for example the growth hormone, might serve as the regulatory mechanism, but this hypothesis seems unlikely since the urinary titers of salt-retaining activity in panhypopituitarism are found to be within approximately the normal range (2, 34).

Tyler, Samuels, and their co-workers (23–25) have proposed that hepatic inactivation of hydrocortisone may be of great importance as a supplementary mechanism, in explaining the variations in blood 17-hydroxycorticosteroid levels occurring both in the "steady state" and under conditions of stress. In accordance with this view, the evidence from the present study suggests the possibility that aldosterone likewise may accumulate in abnormal amounts in disease states as a result of decreased liver inactivation as well as increased adrenal production and release.

The adrenal cortex is currently regarded as an organ primarily concerned with homeostasis of the internal environment. For the purpose of effecting this complex type of physiological control, the gland is capable of releasing several steroid compounds into the blood stream, each possessing specialized and characteristic biological effects. The over-all hormonal response to the adrenal secretion in the intact animal may therefore be expected to be an algebraic summation of the individual actions of its active components. Metabolic modification of all of these compounds by the liver, under a wide variety of environmental conditions may represent an additional refinement of the control mechanism to the end of preventing an abnormal accumulation of biologically potent hormones. Viewed against this background, the increased amounts of salt-retaining hormone occurring in edematous states may be interpreted either as an unfortunate consequence of impaired liver function, or as a continually acting compensatory mechanism directed toward the homeostasis of electrolyte and osmolarity conditions in the circulation, in the face of pathological factors which cause a continuous loss of sodium and water into the extra-vascular space. Thus control receptors in the circulation might fail to be activated and adrenal over-production of aldosterone would be superimposed upon impaired hepatic inactivation as a cause of abnormal sodium retention by the kidney. Choice between these two hypotheses is impossible on the basis of present experimental evidence.

CONCLUSIONS

1. Surviving, actively respiring rat liver slices incubated with urinary sodium-retaining factor (aldosterone) are capable of completely inactivating the biological potency of the hormone.

2. Anaerobic conditions impair this inactivating capacity of liver slices.

3. Kidney slices produce insignificant inactivation of the salt-retaining hormone.

4. Under these experimental conditions, human liver slices manifest a capacity for inactivation of salt-retaining hormone similar to that of rat tissues.

5. Hepatic inactivation and removal of "active" steroid hormones, including aldosterone, may serve as a supplementary homeostatic mechanism, a derangement of which, through impaired liver function, may lead to abnormal accumulation of hormone in blood, tissues and urine.

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EVALUATION OF RESPIRATORY COMPENSATION IN METABOLIC ALKALOSIS [1, 2]

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It has been postulated that a metabolic alkalosis could be partially compensated by a decrease in respiratory exchange consequent to inhibition of the respiratory center by an elevated blood pH (1–5). The extent to which compensation may occur has been predicted to be within the limits imposed by alterations in blood oxygen and pCO_2 (2, 5). The calculated respiratory exchange which has been predicted with alterations in blood pH has been challenged on a theoretical basis (6), and the actual occurrence of significant respiratory compensation in metabolic alkalosis has been denied on the basis that no overt decrease in respiratory exchange was noted acutely following the administration of $NaHCO_3$ (7–9). However, there are few data on actual respiratory exchange or plasma pCO_2 during acute metabolic alkalosis in unanesthetized animals or human subjects, and the published data are insufficient to permit an evaluation of respiratory compensation in chronic metabolic alkalosis. Accordingly, the studies reported here were carried out on patients and dogs to determine the extent of respiratory compensation in both acute and chronic metabolic alkalosis. The data suggest that respiratory compensation is minimal in the majority of patients and dogs with metabolic alkalosis as produced by bicarbonate infusion, gastric drainage of chloride or potassium deficiency.

METHODS

The data presented here include analyses of blood pH, plasma carbon dioxide content and calculated plasma pCO_2 as determined on 34 patients with metabolic alkalosis and 19 normal patients. Clinically, the types of metabolic alkalosis which were encountered in the patients included: 1) potassium deficiency alkalosis resulting from post-operative deficits, inadequate intake or hyperadrenocorticism, 2) hypochloremic alkalosis as instigated by gastric drainage or vomiting secondary to upper small bowel obstruction, and 3) alkalosis resulting from exogenous lactate or bicarbonate administration. In the patients presented, the calculated plasma pCO_2 and measured pH were used as an index to determine the extent of respiratory compensation. Carbon dioxide content and pH in these patients were analyzed either on arterialized venous blood drawn without stasis or directly on arterial blood.[3] In some of the patients, venous blood was drawn without stasis, but was not previously arterialized. The blood was drawn for analysis at a time when the patients had not received sedation. The data from reports in the literature which are incorporated represent analyses which were carried out on unanesthetized dogs or patients with no deducible evidence of pulmonary dysfunction.

The experimental studies were carried out, in the present study, on unanesthetized dogs, and included measurements of minute volume, respiratory rate, alveolar ventilation, plasma carbon dioxide content and blood pH previous to and during a period of metabolic alkalosis induced either by infusion of sodium bicarbonate or by gastric drainage of chloride.

The animals were trained to stand quietly in a loosely restraining dog sling during the period of metabolic alkalosis. Gastric contents were collected from dogs by an indwelling catheter inserted through a permanent fistula created by the insertion of a biflanged steel button extending from the stomach to the abdominal wall.

In nine alkalotic dogs Sodium Pentothal® was given and the animals were then artificially ventilated to induce a decrease in alveolar ventilation which was just sufficient to reduce the blood pH to normal, and thus "compensate" the metabolic alkalosis. In these animals

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[2] We are indebted to Dr. Rulon W. Rawson for his cooperation and helpful suggestions in carrying out these studies and to Dr. Robert F. Pitts for reviewing this work.

[3] The inherent error in utilizing venous blood for analysis of pH and carbon dioxide must be considered since it is well known that the pH of venous blood is lower than in arterial blood, whereas the carbon dioxide content is higher. This error was partly obviated by using arterial blood or arterializing venous blood in some of the patients. However, the finding that the pH and pCO_2 did not reflect significant respiratory compensation even with the use of venous blood strengthens the premise, since it would be expected that the error would be in the opposite direction from that reported here.

arterial oxygen content and oxygen capacity were analyzed.

Arterial blood samples were drawn from an indwelling arterial catheter, and the chemical analyses of carbon dioxide and pH carried out according to previously described methods (10). Blood oxygen content and capacity were determined in duplicate by the method of Van Slyke and Neill (11). The animals were breathing room air throughout and expired gases were collected in a Tissot Gasometer through a set of valves and a snugly fitted rubber face mask which had a dead space of approximately 35 cc. Gas analyses were carried out according to the method of Scholander (12). Physiological dead space was calculated from the Bohr equation as modified by Rahn (13). It was assumed in these calculations that arterial pCO_2 was equal to the effective alveolar pCO_2 (14). Effective alveolar ventilation was then calculated as follows: Alveolar ventilation = (Tidal volume − Dead space) × Respiratory rate.

RESULTS

Figure 1 summarizes the plasma pH and pCO_2 at varying levels of carbon dioxide content in 21 unanesthetized dogs during metabolic alkalosis produced either by alkali infusion or gastric drainage of chloride. The published data of Pitts and Lotspeich (15) are included for comparison. As indicated by this overall summary, the blood pH increased, whereas the plasma pCO_2 remained within the normal range despite elevations in total carbon dioxide.

Although the unchanged plasma pCO_2 suggests that pulmonary ventilation is not significantly decreased in metabolic alkalosis, this was further confirmed by direct measurements of minute volume in nine of the animals studied. The data from these nine dogs are summarized in Table I, and show that there was not a consistent decrease in minute volume during metabolic alkalosis. Similarly, the effective alveolar ventilation during metabolic alkalosis was not consistently changed. In some of the animals alveolar ventilation decreased and in others it increased during the pe-

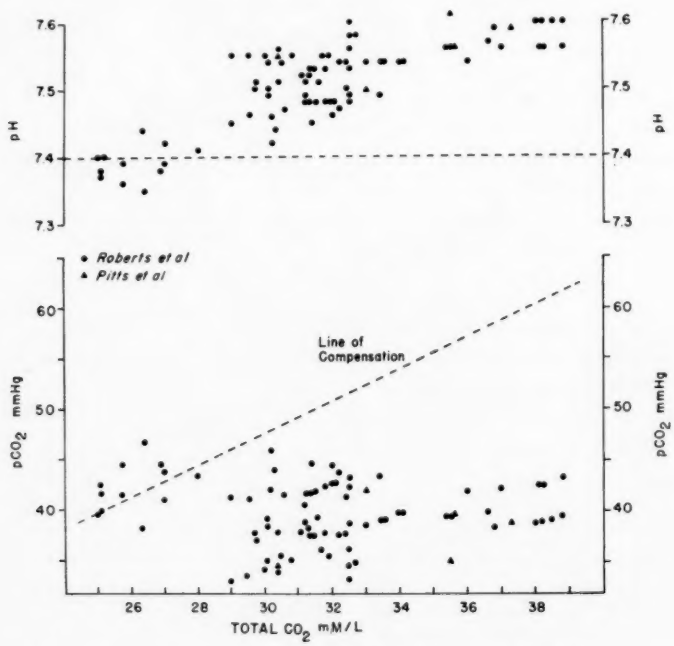

FIG. 1. BLOOD pH AND PLASMA pCO_2 IN RELATION TO TOTAL CARBON DIOXIDE CONTENT IN ALKALOTIC AND NORMAL DOGS

The line of compensation is calculated from a theoretical consideration of the change in plasma pCO_2 which would occur if the metabolic alkalosis were completely compensated, to produce a blood pH of 7.4. The data of Pitts and Lotspeich (15) are also plotted for comparison.

TABLE I

Average respiratory minute volume in dogs previous to and during metabolic alkalosis *

Type of metabolic alkalosis	Minute resp. volume *L./min.*	pH	Plasma CO_2 *mM/L.*	pCO_2 *mm. Hg*
1)				
Control	3.12	7.35	24.3	43
Alkalosis (Gastric drainage)	3.67	7.50	34.4	44
2)				
Control	2.89	7.39	27.06	44
Alkalosis (Gastric drainage)	2.9	7.47	30.58	41
3)				
Control	5.5	7.4	24.66	39
Alkalosis ($NaHCO_3$ infusion)	8.0	7.65	45.9	42
4)				
Control	5.62	7.25	19.7	43
Alkalosis ($NaHCO_3$ infusion)	5.60	7.56	37.2	41
5)				
Control	3.7	7.43	27.2	40
Alkalosis ($NaHCO_3$ infusion)	6.6	7.63	43.05	41
6)				
Control	5.6	7.37	24	41
Alkalosis ($NaHCO_3$ infusion)	6.0	7.51	31.63	39
7)				
Control	4.4	7.31	21.0	41
Alkalosis ($NaHCO_3$ infusion)	3.5	7.56	38.8	43
8)				
Control	4.6	7.40	23.14	37
Alkalosis ($NaHCO_3$ infusion)	4.9	7.57	38.6	42
9)				
Control	2.8	7.35	29.9	53
Alkalosis (Gastric drainage)	2.9	7.48	32.1	43

* The respiratory volume represents an average of two to three determinations in the control period and four to six determinations during the alkalotic interval. The plasma values represent single determinations in the control period and at the end of the alkalotic period.

riod of study. This is illustrated in Figure 2, which shows the alveolar ventilation ratio in six of the animals studied.[4]

Table II summarizes the maximal changes in arterial oxygen saturation during a period when alveolar ventilation was reduced sufficiently by anesthesia and artificial ventilation to completely compensate the metabolic alkalosis. In all of the animals arterial oxygen saturation decreased during the period of artificial compensation. In the five animals who were ventilated sufficiently to restore pH and pCO_2 to alkalotic levels it was noted that arterial oxygen saturation was restored toward control alkalotic values. We have interpreted this observation to indicate that the anesthesia *per se* had no effect on arterial oxygen saturation and that alterations noted during anesthesia were secondary to decreased pulmonary exchange.

[4] The change in respiration necessary to compensate a metabolic alkalosis could be slight, and may not be detected by the usual methods of measurement. Since a knowledge of the plasma CO_2 tension can be utilized to give information regarding the overall ability of the lung to exchange CO_2 (16), this is probably a more reliable "index" of the degree to which respiratory compensation occurs in metabolic alkalosis.

Evaluation of respiratory compensation in patients with metabolic alkalosis

Figure 3 summarizes the plasma pCO_2 and blood pH in relation to total carbon dioxide content of the plasma in 34 patients studied by the authors. For comparative purposes published data of other authors (17–23) are included. The majority of patients shown demonstrated an elevation of blood pH, while pCO_2 was little changed from the normal range. This was true at all levels of plasma carbon dioxide content and regardless of the type of alkalosis encountered. Significant compensation was achieved by only five of the patients summarized from the literature. The remaining forty-five patients displayed minimal or no respiratory

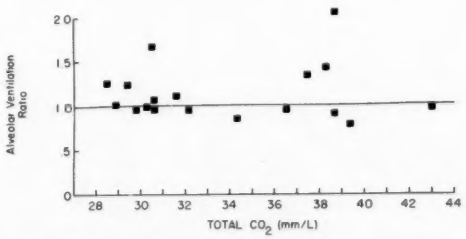

FIG. 2. ALVEOLAR VENTILATION RATIO IN DOGS DURING METABOLIC ALKALOSIS

The alveolar ventilation ratio was calculated as follows:

$$\frac{\text{Alveolar ventilation during metabolic alkalosis (L./min.)}}{\text{Control alveolar ventilation (L./min.)}}$$

Two measurements of alveolar ventilation were carried out on the animals during the control period and three measurements were carried out during the period of metabolic alkalosis at varying levels of plasma CO_2 content and at a time when the animals had been stabilized at the indicated levels for 30 to 40 minutes.

TABLE II

Arterial oxygen saturation during the interval of metabolic alkalosis and following artificially induced respiratory compensation *

	Plasma			Arterial oxygen % saturation
	Carbon dioxide *mM/L.*	pH	pCO_2 *mm. Hg*	
1)				
Alkalosis	38.8	7.56	43	91
Anesthesia	Reduced ventilation			
	36.8	7.38	61	78
2)				
Alkalosis	38.8	7.63	37	97
Anesthesia	Reduced ventilation			
	49	7.38	81	73
Anesthesia	Increased ventilation			
	44.9	7.6	46	99
3)				
Alkalosis	38.6	7.57	42	94
Anesthesia	Reduced ventilation			
	52.3	7.41	81	50
Anesthesia	Increased ventilation			
	53.8	7.54	63	89
4)				
Alkalosis	32.4	7.6	33	95
Anesthesia	Reduced ventilation			
	36.7	7.4	58	84
Anesthesia	Increased ventilation			
	35.4	7.56	39	96
5)				
Alkalosis	30.1	7.54	35	98
Anesthesia	Reduced ventilation			
	31.6	7.41	49	87
6)				
Alkalosis	31.63	7.51	39	99
Anesthesia	Reduced ventilation			
	39.9	7.38	66	72
7)				
Alkalosis	31.3	7.52	38	96
Anesthesia	Reduced ventilation			
	37.8	7.40	60	82
Anesthesia	Increased ventilation			
	36.0	7.56	40	97
8)				
Alkalosis	31.58	7.51	39	93
Anesthesia	Reduced ventilation			
	33.6	7.41	52	86
Anesthesia	Increased ventilation			
	32.19	7.54	37	93.3
9)				
Alkalosis	44.4	7.61	44	97
Anesthesia	Reduced ventilation			
	46.8	7.42	71	47

* Arterial O_2 was studied in five of the animals who were breathed at a rate sufficient to return plasma pH and pCO_2 toward control alkalotic values. The values shown were determined at a time when the pH had remained constant for an interval of 20 to 40 minutes.

compensation as indicated by the failure of plasma pCO_2 to approach the "line of compensation."

DISCUSSION

The data presented cast doubt on the theoretical prediction that metabolic alkalosis is significantly compensated by an increase in plasma carbon dioxide resulting from a decrease in alveolar ventilation (1–5). In alkalotic dogs this failure was apparent directly from measurements of pulmonary ventilation and indirectly by calculations of plasma pCO_2. The finding that the pH was elevated and plasma pCO_2 was little changed from normal in the majority of patients presented, furnished indirect evidence that respiratory compensation is usually minimal in metabolic alkalosis. Conceivably, the interpretations of alveolar ventilation, plasma pH and pCO_2 during acute bicarbonate administration are subject to criticism on the basis that this circumstance does not represent a "steady state" (*2*). However, the similarity of the findings in the acute experiments, chronic potassium depletion alkalosis and hypochloremic alkalosis weakens this criticism.

The prediction that a decreased respiratory exchange occurs in metabolic alkalosis has presumably been based on the concept that extracellular pH is one factor which is instrumental in the chemical control of respiration. It is generally agreed that a decrease in plasma pH results in an increased alveolar ventilation and is partially responsible for compensating a metabolic acidosis (1–5, 24). For this reason, it has been assumed that an elevation of plasma pH would result in the opposite sequence of events. However, any speculation regarding the role of blood pH in the regulation of respiration must be tempered by a consideration of other factors which are also influential in the chemical control of respiration (1, 2, 4, 6, 9). The evidence indicating the role of blood oxygen and pCO_2 in this respect has led to the hypothesis that respiration is controlled by a variety of interrelated factors and that net alveolar exchange will be the resultant of these integrated forces (*2*, 5, 6). The studies reported here augment this thesis and suggest that significant respiratory compensation in metabolic alkalosis may be limited in part by the oxygen requirements of the organism. This consideration is strengthened

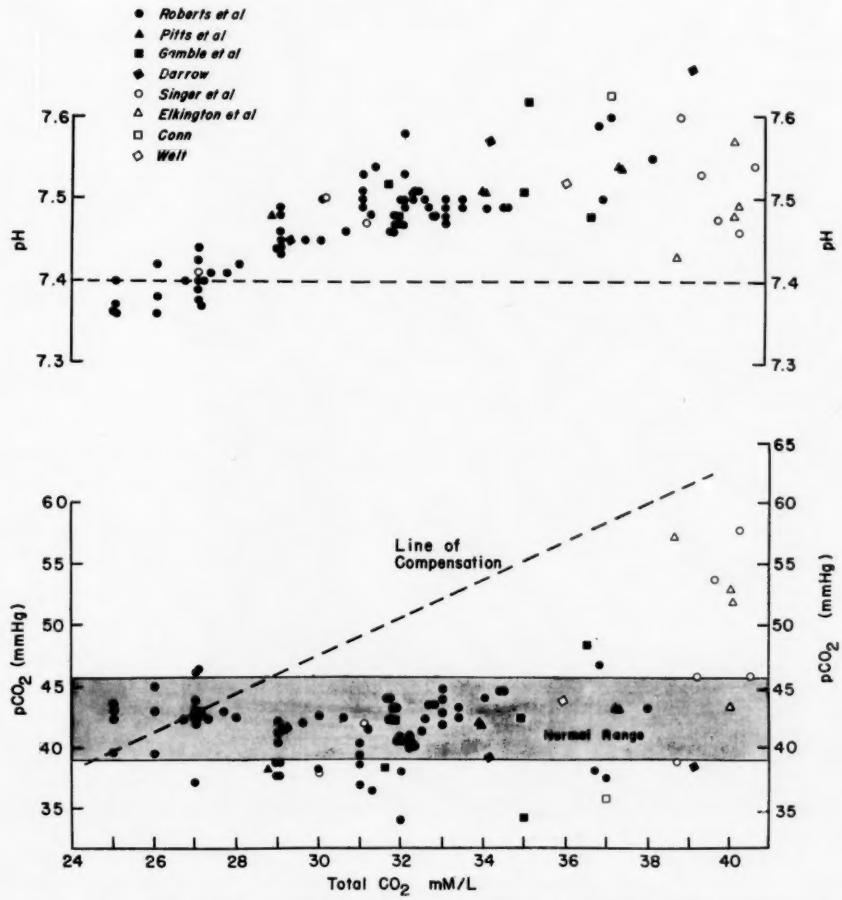

FIG. 3. BLOOD pH AND PLASMA pCO_2 IN RELATION TO TOTAL CARBON DIOXIDE CONTENT IN ALKALOTIC AND NORMAL PATIENTS

The plotted points represent from one to three separate determinations analyzed in duplicate on each patient. The data are also plotted from patients studied by Pitts (17), Elkinton, Squires, and Crosley (21), Conn (22), Welt (23), Darrow (19), and Gamble, Fahey, Appleton, and Maclachlan (20). The data of Singer, Clark, Barker, Crosley, and Elkinton (18) which have been plotted, represent one pCO_2 for each patient taken at the height of alkalosis (*i.e.*, highest total CO_2 content). Data from one patient reported by these authors have not been plotted since this patient had a pCO_2 which was already abnormally elevated in the central period (R. L.) (1). The stippled area indicates the range of normal values for plasma pCO_2 tension.

by the observation that a decrease in arterial oxygen saturation was found in the animals following a decrease in alveolar ventilation sufficient to completely compensate the metabolic alkalosis. However, the data do not permit an evaluation of the extent to which respiratory compensation could theoretically occur, without significant anoxia.

CONCLUSIONS

1. Respiratory compensation in metabolic alkalosis was found to be minimal in dogs and in the majority of patients shown.

2. In alkalotic dogs no consistent decrease in alveolar ventilation or minute volume was measured.

3. It has been suggested that changes in arterial oxygen saturation may limit respiratory compensation in metabolic alkalosis.

Addendum

Since this paper was submitted it has been reported, on the basis of changes in plasma pCO_2 that respiratory compensations occur in metabolic alkalosis (Bramlitt, E., and Hardy, J. D., Surgical Forum Program, p. 111, 41st Annual Clinical Congress, American College of Surgeons, 1955). Calculations utilizing either the Van Slyke nomogram or the Henderson-Hasselbalch equation and these authors published data of plasma carbon dioxide combining power (which closely approximates carbon dioxide content) and blood pH indicates that the average pCO_2 of these patients was 34.5 mm. Hg in the control period and 35.4 mm. Hg during the alkalotic period. This is a change of doubtful significance and well within the range of experimental error.

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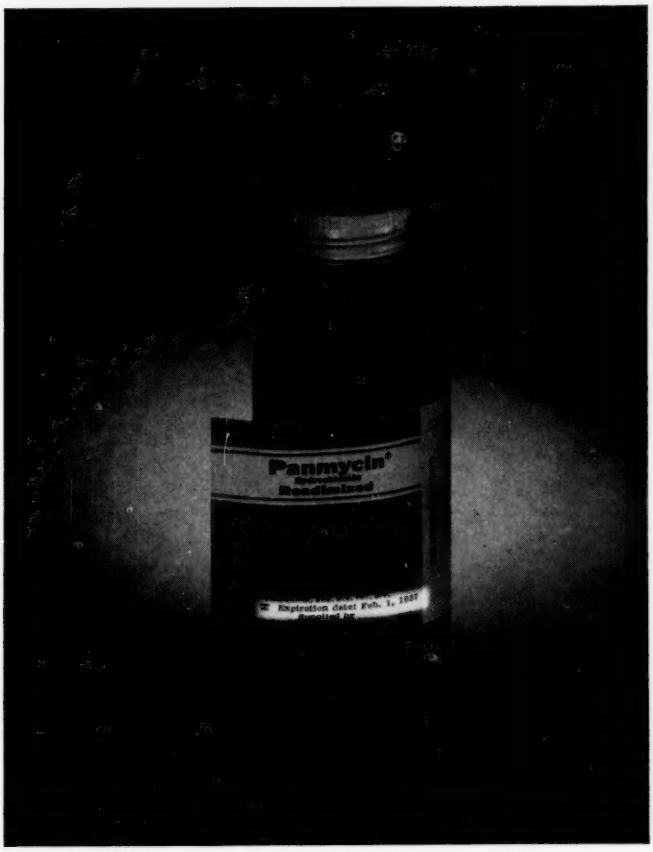

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